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Volumes 60–73.
Volumes 75–77.
Index to Volumes 11–20.
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CONTENTS OF VOLUME 77, No. 3, 1960

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PAGE

Metabolism of amines. 3. The degradation and synthesis of γ-aminobutyric acid by *Pseudomonas aeruginosa*. By U. BACHRACH 417

Cobalamin and the enzymic formation of a factor concerned in the synthesis of methionine by *Escherichia coli*. By J. R. GUEST and D. D. WOODS 422

The metabolism of C_2 compounds in micro-organisms. 4. Synthesis of cell materials from acetate by *Aspergillus niger*. By J. F. COLLINS and H. L. KORNBERG 430

The metabolism of C_2 compounds in micro-organisms. 5. Biosynthesis of cell materials from acetate in *Escherichia coli*. By H. L. KORNBERG, P. J. R. PHIZACKERLEY and J. R. SADLER . . 438

The determination of cholesterol and coprosterol in faecal lipids. By T. GERSON . . . 446

5-Fluorouracil and mucopeptide biosynthesis by *Staphylococcus aureus*. By H. J. ROGERS and H. R. PERKINS 448

A spectrophotometric study of the reaction catalysed by serine transhydroxymethylase. By R. L. BLAKLEY 459

Purification and properties of a protease from the gut of *Etroplus suratensis*. By S. SUNDARAM and P. S. SARMA 465

A protein from leaves catalysing the reduction of metmyoglobin and triphosphopyridine nucleotide by illuminated chloroplasts. By H. E. DAVENPORT 471

The reaction of mustard gas with nucleic acids *in vitro* and *in vivo*. By P. BROOKES and P. D. LAWLEY 478

The simple monosubstituted guanidines of mammalian brain. By J. P. BLASS 484

The incorporation of radioactive amino acids into proteins of the microsome fraction of guinea-pig liver at very short time intervals after administration. By J. L. SIMKIN and C. R. SUTTON 489

Studies in detoxication. 84. The metabolism of [^{14}C]aniline in the rabbit and other animals. By D. V. PARKE 493

The chemical composition and structure of the cell wall of *Hydrodictyon africanum* Yaman. By D. H. NORTHCOTE, K. J. GOULDING and R. W. HORNE. (With three plates) . . . 503

The determination of amino sugars in the presence of amino acids and glucose. By C. CESSI and F. PILIEGO 508

Metabolism of bladder carcinogens. 3. The metabolic path of 2-[8-^{14}C]naphthylamine in several animal species. By M. W. GOLDBLATT, A. F. HENSON and A. R. SOMERVILLE . . . 511

A graphical method for the rapid determination of sedimentation coefficients. By R. MARKHAM 516

The reactions of some aromatic *C*-nitroso compounds with haemoglobin. By Q. H. GIBSON . 519

The estimation of phospholipase A activity in aqueous systems. By W. L. MAGEE and R. H. S. THOMPSON 526

The fractionation of phosphate esters on ion-exchange resin by a new system of pH-gradient elution. By H. E. WADE 534

β-Pyrazol-1-ylalanine, an amino acid from water-melon seeds (*Citrullus vulgaris*). By F. F. NOE and L. FOWDEN 543

Appendix.—Nuclear magnetic resonance spectrum of β-pyrazol-1-ylalanine. By J. H. RIDD and R. F. M. WHITE 546

Frequencies of nucleotide sequences in deoxyribonucleic acids. By K. BURTON 547

Levels and distribution of the acid-soluble ribonucleotides in the mammary glands of pregnant and lactating rats. By D. Y. WANG and A. L. GREENBAUM 552

Synthesis of protein in a purine-requiring *Escherichia coli* infected with bacteriophage T2. By L. I. FESSLER, M. V. KELEMEN and K. BURTON 558

The reaction of carbohydrases with phenolic glucosides. By J. D. ANDERSON, L. HOUGH and J. B. PRIDHAM 564

The acid-soluble nucleotides of codling (*Gadus callarias*) muscle. By N. R. JONES and J. MURRAY 567

Carnosine–anserine synthetase of muscle. 4. Partial purification of the enzyme and further studies of β-alanyl peptide synthesis. By J. J. STENESH and T. WINNICK 575

PAGE

The fractionation of urine colloids on anion-exchange cellulose. By A. J. ANDERSON, M. H. LEPPER and R. J. WINZLER 581

The mode of recovery of cholinesterase activity *in vivo* after organophosphorus poisoning. 1. Erythrocyte cholinesterase. By L. C. BLABER and N. H. CREASEY 591

The mode of recovery of cholinesterase activity *in vivo* after organophosphorus poisoning. 2. Brain cholinesterase. By L. C. BLABER and N. H. CREASEY 597

The estimation of the available lysine in animal-protein foods. By K. J. CARPENTER 604

The intracellular distribution of glycolytic and other enzymes in rat-brain homogenates and mitochondrial preparations. By M. K. JOHNSON 610

The purification and properties of the tyrosine-2-oxoglutarate transaminase of *Saccharomyces cerevisiae*. By S. SENTHESHANMUGANATHAN 619

Fatty acid composition of phospholipids from subcellular particles of rat liver. By M. G. MACFARLANE, G. M. GRAY and L. W. WHEELDON 626

Improved fractionations of arginine-rich histones from calf thymus. By E. W. JOHNS, D. M. P. PHILLIPS, P. SIMSON and J. A. V. BUTLER 631

Polarographic measurement of oxygen uptake by pea-root mitochondria. By R. G. STICKLAND . 636

Proceedings of The Biochemical Society

Chemical aspects of polynucleotide synthesis. By A. M. MICHELSON 13P

Mode of action of polynucleotide phosphorylase. By M. GRUNBERG-MANAGO 13P

Studies on the biosynthesis of ribonucleic acid. By R. H. BURDON 14P

Pyrophosphorolysis of soluble ribonucleic acid. By E. S. CANELLAKIS 14P

Effect of phosphodiesterase on the capacity of soluble ribonucleic acid to accept amino acids. By E. HERBERT 14P

Some characteristics of the phosphorylating kinases of neoplastic and normal cells. By P. BIANCHI, J. A. V. BUTLER, A. R. CRATHORN and K. V. SHOOTER 15P

Studies on the biosynthesis of deoxyribonucleic acid. By R. M. S. SMELLIE 15P

Biosynthesis of bacteriophage deoxyribonucleic acid. By L. I. FESSLER 16P

Phosphorylation of some analogues of fructose by yeast hexokinase. By J. R. BURT 16P

The nature of 'cholesterol' in normal human-blood plasma. By M. GREIG and R. P. COOK . 16P

Inhibition of incorporation of ^{14}C-labelled purines into nucleic acids by a substituted pyrimidine. By B. A. NEWTON 17P

Sites of synthesis of a nucleic acid and a protein in infected Krebs-2 ascites tumour cells. By A. J. D. BELLETT and A. T. H. BURNESS 17P

Mechanism of conjugation of *o*-aminophenol in plants and an insect. By G. J. DUTTON and A. M. DUNCAN 18P

Mechanism of glucuronide synthesis in skin. By I. H. STEVENSON and G. J. DUTTON . . 19P

Transfer of soluble ribonucleic acid to microsomes in an *in vitro* system from rat liver. By C. J. SMITH and E. REID 19P

p-Hydroxyphenylacetic acid *O*-sulphate: a metabolite of tyrosine *O*-sulphate in the rat. By K. S. DODGSON, G. M. POWELL, F. A. ROSE and N. TUDBALL 20P

Changes in two microsomal components accompanying cell differentiation. By U. E. LOENING . 20P

Inhibitor studies on the time sequence of nucleic acid and protein synthesis in mammalian cells. By J. PAUL and A. HAGIWARA 21P

'Conjugate' formation during the metabolism of [4-^{14}C]progesterone by female-rat liver *in vitro*. By B. A. COOKE and W. TAYLOR 21P

Progesterone metabolism in the cat. By W. TAYLOR and T. SCRATCHERD 22P

Non-enzymic transamination between glycine and glyoxylate. By L. W. FLEMING and G. W. CROSBIE 23P

Photofixation of nitrogen and photoproduction of hydrogen by thiosulphate during bacterial photosynthesis. By D. I. ARNON, M. LOSADA, M. NOZAKI and K. TAGAWA 23P

The effect of protein intake on the ribonucleic acid of liver cell sap. By E. J. T. PROSSER, A. C. MALLINSON and H. N. MUNRO 24P

INDEX OF AUTHORS

	PAGE
Anderson, A. J.	581
Anderson, J. D.	564
Arnon, D. I.	23P
Bachrach, U.	417
Bellett, A. J. D.	17P
Bianchi, P.	15P
Blaber, L. C.	591, 597
Blakley, R. L.	459
Blass, J. P.	484
Brookes, P.	478
Burdon, R. H.	14P
Burness, A. T. H.	17P
Burt, J. R.	16P
Burton, K.	547, 558
Butler, J. A. V.	631, 15P
Canellakis, E. S.	14P
Carpenter, K. J.	604
Cessi, C.	508
Collins, J. F.	430
Cook, R. P.	16P
Cooke, B. A.	21P
Crathorn, A. R.	15P
Creasey, N. H.	591, 597
Crosbie, G. W.	23P
Davenport, H. E.	471
Dodgson, K. S.	20P
Duncan, A. M.	18P
Dutton, G. J.	18P, 19P
Fessler, L. I.	558, 16P
Fleming, L. W.	23P
Fowden, L.	543
Gerson, T.	446
Gibson, Q. H.	519
Goldblatt, M. W.	511
Goulding, K. J.	503
Gray, G. M.	626
Greenbaum, A. L.	552
Greig, M.	16P
Grunberg-Manago, M.	13P
Guest, J. R.	422
Hagiwara, A.	21P
Henson, A. F.	511
Herbert, E.	14P
Horne, R. W.	503
Hough, L.	564
Johns, E. W.	631
Johnson, M. K.	610
Jones, N. R.	567
Kelemen, M. V.	558
Kornberg, H. L.	430, 438
Lawley, P. D.	478
Lepper, M. H.	581
Loening, U. E.	20P
Losada, M.	23P
Macfarlane, M. G.	626
Magee, W. L.	526
Mallinson, A. C.	24P
Markham, R.	516
Michelson, A. M.	13P
Munro, H. N.	24P
Murray, J.	567
Newton, B. A.	17P
Noe, F. F.	543
Northcote, D. H.	503
Nozaki, M.	23P
Parke, D. V.	493
Paul, J.	21P
Perkins, H. R.	448
Phillips, D. M. P.	631
Phizackerley, P. J. R.	438
Piliego, F.	508
Powell, G. M.	20P
Pridham, J. B.	564
Prosser, E. J. T.	24P
Reid, E.	19P
Ridd, J. H.	546
Rogers, H. J.	448
Rose, F. A.	20P
Sadler, J. R.	438
Sarma, P. S.	465
Scratcherd, T.	22P
SentheShanmuganathan, S.	619
Shooter, K. V.	15P
Simkin, J. L.	489
Simson, P.	631
Smellie, R. M. S.	15P
Smith, C. J.	19P
Somerville, A. R.	511
Stenesh, J. J.	575
Stevenson, I. H.	19P
Stickland, R. G.	636
Sundaram, S.	465
Sutton, C. R.	489
Tagawa, K.	23P
Taylor, W.	21P, 22P
Thompson, R. H. S.	526
Tudball, N.	20P
Wade, H. E.	534
Wang, D. Y.	552
Wheeldon, L. W.	626
White, R. F. M.	546
Winnick, T.	575
Winzler, R. J.	581
Woods, D. D.	422

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of *The Biochemical Journal*:

Aspects of the metabolism of glycine and of porphyrins. The second Hopkins memorial lecture. By ALBERT NEUBERGER

An investigation of the egg-white mucoproteins, ovomucoid and ovalbumin. By P. D. BRAGG and L. HOUGH

Characterization of naturally occurring materials which restore excitability to isolated cerebral tissues. By H. MCILWAIN

Migration of histones from the nuclei of isolated cerebral tissues kept in cold media. By L. S. WOLFE and H. MCILWAIN

The metabolism of griseofulvin in mammals. By M. J. BARNES and B. BOOTHROYD

Cardiolipin and other phospholipids in ox liver. By MARJORIE G. MACFARLANE

The amide nitrogen content of gelatins. By J. E. EASTOE, JOAN E. LONG and ALICE L. D. WILLAN

Formation of labelled amino acids by exchange transamination. By R. G. HILLER and D. A. WALKER

The isolation of bradykinin, a plasma kinin from ox blood. By D. F. ELLIOTT, E. W. HORTON and G. P. LEWIS

Preparation of purified 3-hydroxyanthranilic acid oxidase from rat and ox liver. By M. IACCARINO, E. BOERI and V. SCARDI

The metabolism of C_2 compounds in micro-organisms. 6. Synthesis of cell constituents from glycollate by *Pseudomonas* sp. By H. L. KORNBERG and A. M. GOTTO

The biochemistry of epithelia. Enzyme levels in bronchial and tracheal epithelia of smokers and non-smokers and in bronchial carcinoma. By B. SPENCER

The metabolism of isolated rat-liver nuclei. By K. R. REES and G. F. ROWLAND

The reduction of acetoacetate to β-hydroxybutyrate in animal tissues. By R. G. KULKA, H. A. KREBS and L. V. EGGLESTON

Studies on glucosaminidase:

2. Substrates for *N*-acetyl-β-glucosaminidase. By J. BOROOAH, D. H. LEABACK and P. G. WALKER

3. Testicular *N*-acetyl-β-glucosaminidase and *N*-acetyl-β-galactosaminidase. By J. W. WOOLLEN, R. HEYWORTH and P. G. WALKER

Mechanism of transaminase action. By T. OSHIMA and N. TAMIYA

Condensed tannins. 7. Isolation of (−)-7:3′:4′-trihydroxyflavan-3-ol [(−)-fisetinidol], a naturally occurring catechin from black-wattle heartwood. By D. G. ROUX and E. PAULUS

The effect of riboflavin and mepacrine on the metabolism of 5-hydroxytryptamine. By M. H. WISEMAN and T. L. SOURKES

Evidence for the occurrence of uridine diphosphate *N*-acetylglucosamine in crustacean tissues. By MARY R. LUNT and P. W. KENT

Studies in immunochemistry. 18. The isolation and properties of a sialomucopolysaccharide possessing blood-group Le^a specificity and virus-receptor activity. By A. PUSZTAI and W. T. J. MORGAN

The enzymic formation of haem by human and rat tissues. By ANN C. LOCHHEAD and A. GOLDBERG

Studies on glucosaminidase. 4. The fluorimetric assay of *N*-acetyl-β-glucosaminidase. By D. H. LEABACK and P. G. WALKER

Purification and specificity of pancreatic elastase. By M. A. NAUGHTON and F. SANGER

Ultracentrifugal studies of rat, rabbit and guinea-pig serum albumins. By P. A. CHARLWOOD

A colorimetric method for the estimation of monoamine oxidase. By A. L. GREEN and THELMA M. HAUGHTON

The biochemistry of aromatic amines. 8. Synthesis and detection of di(2-amino-1-naphthyl) hydrogen phosphate, a metabolite of 2-naphthylamine in dogs. By E. BOYLAND, C. H. KINDER and D. MANSON

Chromatographic separation of brain lipids: cerebroside and sulphatide. By C. LONG and D. A. STAPLES

On the mechanism of α-oxoglutarate oxidation in *Escherichia coli*. By L. P. HAGER and H. L. KORNBERG

The phospholipids of butter and their effect on blood coagulation. By J. D. BILLIMORIA, R. G. CURTIS and N. F. MACLAGAN

The measurement of aconitase activity in the leaves of various normal and variegated plants. By J. S. D. BACON, M. J. PALMER and P. C. DEKOCK

THE BIOCHEMICAL JOURNAL

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CORRIGENDUM

The utilization of L-cystinyl-L-valine for penicillin biosynthesis

By H. R. V. Arnstein and D. Morris

Volume 76 (1960)

p. 324, column 2, lines 2 and 4
p. 325, column 2, lines 27 and 28

for 0·0171 m-mole *read* 0·0215 m-mole

p. 326, column 2, penultimate line

for 83 μC/m-mole *read* 67 μC/m-mole.

Biochem. J. (1960) **77**, 417

Metabolism of Amines

3. THE DEGRADATION AND SYNTHESIS OF γ-AMINOBUTYRIC ACID BY *PSEUDOMONAS AERUGINOSA**

BY U. BACHRACH

Department of Clinical Microbiology, Hebrew University-Hadassah Medical School, Jerusalem, Israel

(*Received* 9 *May* 1960)

The degradation of spermine and spermidine by various bacteria has been described by Razin, Gery & Bachrach (1959). γ-Aminobutyric acid, an intermediate in these degradations, could be further metabolized by transamination, by washed cell suspensions of *Pseudomonas aeruginosa* and *Mycobacterium smegmatis* (Bachrach, Persky & Razin, 1960). This study deals with the mechanism of γ-aminobutyric acid degradation by cell-free preparations of *P. aeruginosa*, and the reversal of this process.

MATERIALS AND METHODS

Organism. The strain of *P. aeruginosa* used throughout this study was the one described by Razin *et al.* (1959).

Medium. P. aeruginosa was grown on a medium composed of: yeast extract (Difco), 1 g.; K_2HPO_4, 2 g.; KH_2PO_4, 1 g.; $MgSO_4,7H_2O$, 0·2 g.; γ-aminobutyric acid, 3 g.; agar, 15 g.; water, 1 l. The final pH was 7·2.

Cell suspensions and enzyme preparations. Bacteria grown on the medium specified above for 20 hr. at 37° were harvested, washed twice with 0·85% NaCl soln. and kept at 4° until used. Freeze-dried cells were obtained by drying the bacteria over P_2O_5 at 0·3 mm. Hg in a centrifugal freeze-drier (W. Edwards and Co. Ltd., Crawley, Sussex, England). Cell-free preparations were obtained by subjecting washed cell suspensions to the action of a 10 kcyc. Raytheon sonic oscillator for 20 min. The intact cells and debris were removed by centrifuging at 11 000 *g* for 20 min. Coenzyme A was removed by adsorption on Amberlite IRA-400 (Chantrenne, 1951).

Chemicals. γ-Aminobutyric acid, oxaloacetic acid, α-oxovaleric acid, diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN) and coenzyme A (CoA) were the products of Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. α-Oxoglutaric acid was obtained from L. Light and Co. Ltd., Colnbrook, Bucks., England, alcohol dehydrogenase was produced by Worthington Biochemical Corp., Freehold, N.J., U.S.A. and [1:4-^{14}C]-succinic acid was purchased from The Radiochemical Centre, Amersham, Bucks. Succinic semialdehyde was prepared according to Prescott & Waelsch (1946).

Buffer solutions. The phosphate buffers were mixtures of 0·067 M-Na_2HPO_4 and 0·067 M-KH_2PO_4.

Manometric methods. Manometric experiments were carried out as described by Razin *et al.* (1959). The gas phase was H_2 in anaerobic experiments. The dehydrogenase activity of the cell-free preparation was determined by the Thunberg method (Umbreit, Burris & Stauffer, 1957).

* Part 2: Bachrach, Persky & Razin (1960).

Identification of amines. This was carried out by paper chromatography. Butanol–acetic acid–water (50:25:25, by vol.) (Baker, Harborne & Ollis, 1952) was the standard solvent used. The other solvents included: ethanol, 70% (v/v) (Miller & Rockland, 1952); pyridine–acetic acid–water (50:35:15, by vol.) (Decker & Riffart, 1950); 95% ethanol–amyl alcohol–water (70:50:30, by vol.). The papers were sprayed with 0·2% (w/v) ninhydrin in butanol. The amines were quantitatively assayed according to Giri, Radhakrishnan & Vaidyanathan (1952). γ-Aminobutyric acid was also identified by paper chromatography, by the method of Kalyankar & Snell (1957). Dinitrophenyl derivatives of the amines or amino acids were prepared according to Rosenthal & Tabor (1956) and analysed by paper chromatography, the solvent being either butanol saturated with ammonia or a 0·067 M-phosphate buffer, pH 6·0 (Kojima, Mizuno & Miyazaki, 1958).

Identification of aldehydes and keto acids. 2:4-Dinitrophenylhydrazones of the aldehydes or keto acids were prepared and analysed by paper chromatography according to Block, Durrum & Zweig (1958). The solvents used were: butanol–ethanol–0·5 N-NH_3 soln. (70:10:20, by vol.) (El Hawary & Thompson, 1953), and butanol–ethanol (80:20, v/v) (Cavallini, Frontali & Toschi, 1949). The papers were sprayed with 2% (w/v) KOH in ethanol.

Spectrophotometric analysis. Reduced DPN or TPN were quantitatively determined by the spectrophotometric method (Racker, 1950), with a Unicam SP. 500 spectrophotometer. Proteins were quantitatively estimated by the spectrophotometric method (Layne, 1957).

Identification and assay of labelled compounds. γ-Aminobutyric acid or succinic semialdehydes were identified by paper chromatography and the radioactive areas located on the paper were counted either directly with a mica end-window Geiger–Müller tube, or eluted with water and assayed for radioactivity on planchets with an end-window, lead-shielded Geiger–Müller tube (Tracerlab).

RESULTS

Transamination of γ-aminobutyric acid. γ-Aminobutyric acid was oxidized slowly by washed cells of *P. aeruginosa*. Its oxidation by freeze-dried cells was even slower. Cell-free preparations metabolized γ-aminobutyric acid in the presence of various keto acids. Table 1 shows that oxaloacetate and α-oxovalerate both participated in the transamination, as shown with α-oxoglutarate and pyruvate (Bachrach *et al.* 1960). The pH optimum for the transamination of γ-aminobutyric acid with

α-oxoglutarate by cell suspensions was 7·6 (Fig. 1). The succinic semialdehyde produced from γ-aminobutyric acid was identified by paper chromatography, as described by Bachrach *et al.* (1960).

Metabolism of γ-aminobutyric acid by cell-free preparations. Cell-free preparations did not oxidize γ-aminobutyric acid and the addition of boiled debris did not result in a marked oxygen uptake. On the other hand, methylene blue was reduced anaerobically by cell-free preparations in the presence of γ-aminobutyric acid and α-oxoglutarate. Reduction of TPN occurred when it was added to a reaction mixture containing cell-free preparation, γ-aminobutyric acid and α-oxoglutarate, as shown by the characteristic appearance of the 340 mμ band. DPN was slowly reduced under similar conditions (Fig. 2). With succinic semialdehyde as substrate, the rate of reduction was higher. The oxidation of succinic semialdehyde seems to require the presence of some cofactors. A cell-free preparation which was dialysed overnight against 4 l. of distilled water at 4° retained its ability to transaminate γ-aminobutyric acid, whereas no marked reduction of TPN occurred in the presence of succinic semialdehyde. The treatment of a cell-free preparation with Amberlite IRA-400, which is known to remove CoA, did not affect transamination, but the reduction of TPN was markedly inhibited (Fig. 3).

The interaction between succinic semialdehyde and TPN or DPN should give rise to succinic acid. No succinate could, however, be detected in the reaction mixture by paper chromatography. Succinate might be formed as an intermediate product, without being accumulated, and an attempt was made to demonstrate its participation in the metabolism of γ-aminobutyric acid by the reversal of the degradation process. Succinic acid (100 μmoles) was incubated for 60 min. with a cell-free preparation of *P. aeruginosa* (approx. 4 mg. of protein) in the presence of 20 μmoles of glutathione, 10 μmoles of magnesium sulphate, 1 μmole of DPN, 0·3 ml. of ethanol, 100 μg. of alcohol dehydrogenase and 1 ml. of 0·067 M-phosphate buffer, pH 7·2. Succinic semialdehyde was shown to be formed by paper chromatographic analysis. The semialdehyde formed was converted into γ-aminobutyric acid in the presence of glutamate or α-alanine. The identity of γ-aminobutyric acid in all these experiments was confirmed by paper chromatography with four different solvents, and by

Table 1. *Transamination of γ-aminobutyric acid by cell-free preparations in the presence of various keto acids*

Each Warburg vessel contained 20 μmoles of γ-aminobutyric acid, 20 μmoles of keto acids, 1 ml. of cell-free preparations (approx. 4 mg. of protein) and 1 ml. of 0·067 M-phosphate buffer, pH 7·6, in a total volume of 3·2 ml. Incubation was in H_2 at 37° for 200 min. The amino acids produced from the corresponding keto acids were determined by quantitative paper chromatography.

Keto acids added	Amino acids produced (μmoles)	
α-Oxoglutarate	Glutamate	8·0
Pyruvate	Alanine	5·5
Oxaloacetate	Aspartate	4·2
α-Oxovalerate	Valine	1·6

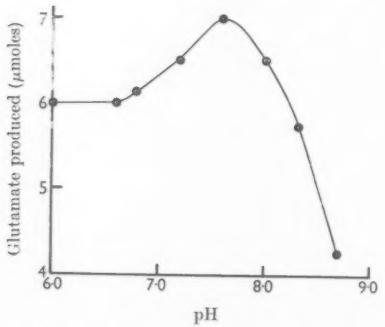

Fig. 1. Effect of pH on the transamination of γ-aminobutyric acid by cell suspensions. Each Warburg vessel contained 10 μmoles of γ-aminobutyric acid, 10 μmoles of α-oxoglutarate, 1 ml. of bacterial suspensions (approx. 5 mg. dry wt.) and 1 ml. of 0·067 M-phosphate buffer, pH 7·3, in a total volume of 3·2 ml. Incubation was in H_2 at 30° for 200 min. The amount of glutamate produced was determined by quantitative paper chromatography.

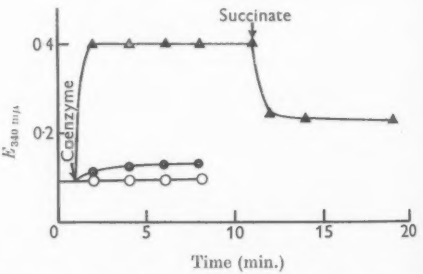

Fig. 2. Oxidation of succinic semialdehyde in the presence of pyridine nucleotides. The reaction mixture contained: 20 μmoles of γ-aminobutyric acid, 20 μmoles of α-oxoglutarate, 2·5 ml. of cell-free preparations (approx. 4 mg. of protein) and 1 ml. of 0·067 M-phosphate buffer, pH 7·3. After 30 min. incubation at 37°, water (○) or 2 μmoles of TPN (▲) or DPN (●) was added to 1 ml. of reaction mixture and examined spectrophotometrically. Succinate (25 μmoles) was added as indicated after another 10 min.

Table 2. *Formation of* ^{14}C*-labelled compounds by reduction of* [^{14}C]*succinate*

The reaction mixture contained 15 μmoles of γ-aminobutyric acid, 15 μmoles of α-oxoglutarate, 2 ml. of cell-free preparations (approx. 5 mg. of protein), and 1 ml. of 0·067 M-phosphate buffer, pH 7·3. After incubation for 30 min. at 37° in air, 2 μmoles of TPN, 50 μmoles of [^{14}C]succinate (3×10^5 counts/min.), 10 μmoles of $MgSO_4$ and 20 μmoles of glutathione were added. The reaction mixture was kept at 37° for 20 min. and examined by paper chromatography. The corresponding spots were eluted and assayed for radioactivity.

Compound formed	Solvent system	Radioactivity (counts/min./ml. of reaction mixture)
Succinic semialdehyde	Butanol–ethanol–ammonia	1 819
γ-Aminobutyric acid	Ethanol–amyl alcohol–water	19 780
	Butanol–acetic acid–water	16 340
	70% Ethanol	12 330

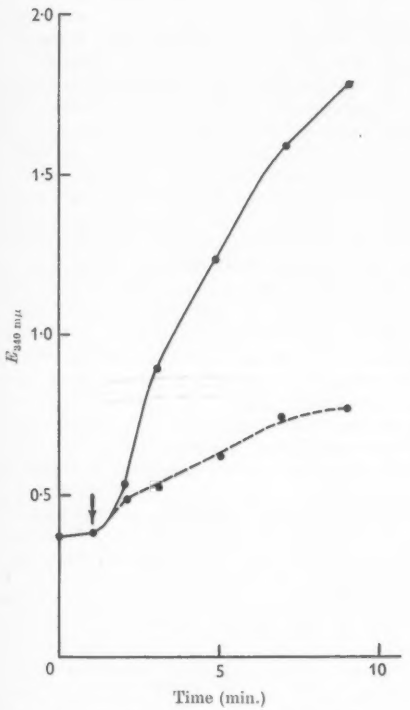

Fig. 3. Oxidation of succinic semialdehyde by various cell-free preparations. The reaction mixture contained: 10 μmoles of γ-aminobutyric acid, 10 μmoles of α-oxoglutarate, 2 ml. of cell-free preparations (approx. 5 mg. of protein) and 1 ml. of 0·067 M-phosphate buffer, pH 7·3. After incubation for 30 min. at 37°, 2 μmoles of TPN were added to the reaction mixtures (indicated by the arrow) and examined spectrophotometrically. —, Cell-free preparation; - - -, cell-free preparation treated with Amberlite IRA-400.

the technique described by Kalyankar & Snell (1957) which is specific for γ- and β-amino compounds. 2:4-Dinitrophenyl derivatives were also prepared and analysed by paper chromatography with two different solvents.

The conversion of succinate into γ-aminobutyric acid was confirmed by the use of [^{14}C]succinate, which was reduced as above with reduced DPN by cell-free preparations of *P. aeruginosa*. The reaction mixture contained 50 μmoles of [^{14}C]-succinic acid (3×10^5 counts/min.), 20 μmoles of glutathione, 10 μmoles of magnesium sulphate, 5 μmoles of DPN, 0·2 ml. of ethanol, 50 μg. of alcohol dehydrogenase, 1 ml. of 0·067 M-phosphate buffer, pH 7·2, and 2 ml. of cell-free preparation (approx. 7·0 mg. of protein); the total volume was 4·0 ml. During the 90 min. incubation at 37°, [^{14}C]succinic semialdehyde was produced. This compound was identified by paper chromatography of the 2:4-dinitrophenylhydrazones with butanol–ethanol–ammonia as solvent. Its radioactivity was 13 624 counts/min.

Reduced TPN, which was more active in the reduction of succinate, was also tested. A cell-free preparation of *P. aeruginosa* was incubated with γ-aminobutyric acid and α-oxoglutarate; TPN was then added to the reaction mixture containing the semialdehyde and formation of reduced TPN was observed. On addition of [^{14}C]succinate to the reaction mixture, now containing reduced TPN, [^{14}C]succinic semialdehyde and γ-amino[^{14}C]-butyric acid were formed. The formation of γ-amino[^{14}C]butyric acid was demonstrated by paper chromatography with three different solvent systems (Table 2). As expected, the addition of succinate to the reaction mixture containing reduced TPN caused the immediate oxidation of the reduced coenzyme (see Fig. 2). Samples of a reaction mixture obtained during the reduction of succinate by reduced DPN were analysed by paper chromatography and showed a ninhydrin-

positive spot of R_F 0·69 with the standard butanol–acetic acid–water solvent system. The compound responsible for this spot did not react as an aldehyde; it was presumed to be a reduction product of γ-aminobutyric acid, probably an amino alcohol. Synthetic 4-aminobutan-1-ol behaved chromatographically like the reduction product and was oxidized by cell suspensions to a compound behaving as γ-aminobutyric acid when examined by paper chromatography with the standard butanol–acetic acid–water solvent system.

Effect of inhibitors on γ-aminobutyric acid degradation. The activity of various inhibitors on the oxidation of γ-aminobutyric acid was examined. Iodoacetate, hydroxylamine and semicarbazide almost completely inhibited the oxidation of γ-aminobutyric acid by freeze-dried cells of *P. aeruginosa* (Table 3). Iodoacetate inhibited the oxidation of γ-aminobutyric acid by washed cell suspensions to a smaller degree. Transamination of γ-aminobutyric acid with α-oxoglutarate was not inhibited by iodoacetate (0·3 mM), as measured by glutamic acid and succinic semialdehyde formation. Arsenite inhibited the γ-aminobutyric acid oxidation by freeze-dried cells by 90% (Table 3). Transamination was not inhibited under these conditions.

Table 3. *Effect of inhibitors on the oxidation of γ-aminobutyric acid by cell suspensions*

Each Warburg vessel contained 10 μmoles of γ-aminobutyric acid, 0·5 ml. of inhibitor, freeze-dried cells (10 mg. dry wt.), and 1 ml. of 0·067 M-phosphate buffer, pH 7·3. The centre well contained 0·2 ml. of 15% (w/v) KOH. Total volume was 3·2 ml. Incubation was in air at 37° for 120 min.

Inhibitor	Concn. (mM)	Inhibition of O_2 uptake (%)
Iodoacetate	0·3	100
Hydroxylamine	0·3	96
Semicarbazide	0·3	92
Arsenite	0·01	90
*iso*Nicotinic acid hydrazide	0·5	62

DISCUSSION

The metabolism of γ-aminobutyric acid by bacterial enzyme systems has been studied by various authors. Because of the stimulatory effect produced by the addition of different keto acids, it is generally believed that the first step in its breakdown is its transamination.

In a strain of *Achromobacter*, α-oxoglutarate and oxaloacetate were the active keto acids (Highashi, Horio & Okunuki, 1957). A strain of *Pseudomonas* described by Scott & Jakoby (1958) was able to transaminate γ-aminobutyric acid in the presence of α-oxoglutarate only. Similar results were obtained by Noe & Nickerson (1958) with a strain of *P. aeruginosa*, isolated from soil, and by *Bacillus pumilus* (Tsunoda & Shiio, 1959). The present investigation confirms these results and supports the view that succinic semialdehyde is formed. The strain of *P. aeruginosa* used in the present study could use α-oxoglutarate, pyruvate, oxaloacetate or α-oxovalerate as keto acids in the transamination reaction. The oxidation of succinic semialdehyde by homogenates of rat brain is catalysed by DPN (Albers & Salvador, 1958), whereas TPN was active in the oxidation of succinic semialdehyde by a *Pseudomonas* strain (Scott & Jakoby, 1958). Our studies indicate that both TPN or DPN may take part in the oxidation of succinic semialdehyde, although the latter was less active. Results presented in this paper indicate that the oxidation of γ-aminobutyric acid by cell-free preparations of *P. aeruginosa* proceeds along the pathway outlined in Fig. 4. γ-Aminobutyric acid is converted into succinic semialdehyde by transamination, in the presence of various keto acids. Succinic semialdehyde is oxidized by TPN or DPN to a compound on the succinate level (perhaps succinyl-CoA). Succinate, which could not be isolated during the oxidation of γ-aminobutyric acid by cell-free preparations of *P. aeruginosa*, accumulated during the oxidation of γ-amino-

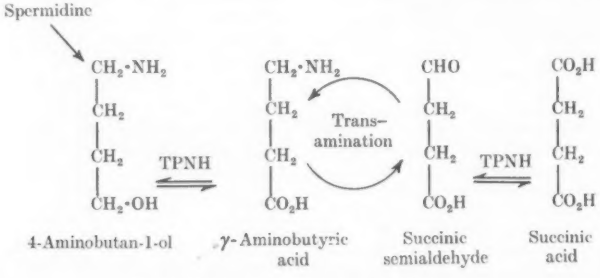

Fig. 4. Reductive amination of succinate.

butyric acid by *M. smegmatis* (Bachrach *et al.* 1960). It is likely that CoA is necessary for the oxidation of succinic semialdehyde, since removal of CoA by treatment of the bacterial preparation with Amberlite IRA-400 resulted in loss of activity. The inhibitory effect exerted by iodoacetate and dialysis points to the possibility that SH groups play a role in the oxidation. Jakoby & Scott (1959) have already demonstrated the importance of SH groups in the oxidation of succinic semialdehyde by *P. fluorescens*. The pathway of γ-aminobutyric acid degradation may be reversed, and succinic acid may serve as a precursor (see Fig. 4). The reductive amination of succinate to γ-aminobutyric acid by cell-free preparations of *P. aeruginosa* not only suggests the participation of succinate in the degradation of γ-aminobutyric acid, but also serves as a new route for the biosynthesis of γ-aminobutyric acid. The reduction of γ-aminobutyric acid to 4-aminobutan-1-ol and the reversal of this process could also contribute to the biosynthesis and degradation of γ-aminobutyric acid. The accumulation of 4-aminobutan-1-ol during the degradation of spermidine by *Serratia marcescens* has already been suggested (Razin *et al.* 1959). The use of inhibitors may be of help in studying the different stages of γ-aminobutyric acid degradation. Thus, iodoacetate did not interfere with the transamination reaction, but inhibited the oxidation of succinic semialdehyde. Similar results were obtained with arsenite, which is known to inhibit the oxidation of aldehydes (Jakoby, 1958).

SUMMARY

1. Extracts of *Pseudomonas aeruginosa* metabolized γ-aminobutyric acid by transamination in the presence of α-oxoglutarate, pyruvate, oxaloacetate or α-oxovalerate.

2. Succinic semialdehyde, produced by transamination, was further oxidized by tri- or diphosphopyridine nucleotide.

3. Succinate was converted into succinic semialdehyde by reduced tri- or di-phosphopyridine nucleotide and yielded γ-aminobutyric acid by transamination.

4. The formation of 4-aminobutan-1-ol by the reductive amination of succinate, and the formation of γ-amino[^{14}C]butyric acid from [^{14}C]succinate, have been described.

REFERENCES

Albers, R. W. & Salvador, R. A. (1958). *Science*, **128**, 359.

Bachrach, U., Persky, S. & Razin, S. (1960). *Biochem. J.* **76**, 306.

Baker, W., Harborne, J. B. & Ollis, W. D. (1952). *J. chem. Soc.* p. 3215.

Block, R. J., Durrum, E. L. & Zweig, G. (1958). *A Manual of Paper Chromatography and Paper Electrophoresis*, p. 235. New York: Academic Press Inc.

Cavallini, D., Frontali, N. & Toschi, G. (1949). *Nature, Lond.*, **164**, 792.

Chantrenne, H. (1951). *J. biol. Chem.* **189**, 227.

Decker, P. & Riffart, W. (1950). *Chemikerztg*, **74**, 261. Cited in *Chem. Abstr.* (1951). **45**, 6258.

El Hawary, M. F. S. & Thompson, R. H. S. (1953). *Biochem. J.* **53**, 340.

Giri, K. V., Radhakrishnan, A. N. & Vaidyanathan, C. S. (1952). *Nature, Lond.*, **170**, 1025.

Highashi, T., Horio, T. & Okunuki, K. (1957). *J. Biochem., Tokyo*, **44**, 735.

Jakoby, W. B. (1958). *J. biol. Chem.* **232**, 89.

Jakoby, W. B. & Scott, E. M. (1959). *J. biol. Chem.* **234**, 937.

Kalyankar, G. D. & Snell, E. E. (1957). *Nature, Lond.*, **180**, 1069.

Kojima, K., Mizuno, K. & Miyazaki, M. (1958). *Nature, Lond.*, **181**, 1200.

Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 451. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Miller, J. M. & Rockland, L. B. (1952). *Arch. Biochem. Biophys.* **40**, 416.

Noe, F. F. & Nickerson, W. J. (1958). *J. Bact.* **75**, 674.

Prescott, B. A. & Waelsch, H. (1946). *J. biol. Chem.* **164**, 331.

Racker, E. (1950). *J. biol. Chem.* **184**, 313.

Razin, S., Gery, I. & Bachrach, U. (1959). *Biochem. J.* **71**, 551.

Rosenthal, S. M. & Tabor, C. W. (1956). *J. Pharmacol.* **116**, 131.

Scott, E. M. & Jakoby, W. B. (1958). *Science*, **128**, 361.

Tsunoda, T. & Shiio, I. (1959). *J. Biochem., Tokyo*, **46**, 1011.

Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*. Minneapolis: Burgess Publishing Co.

Biochem. J. (1960) **77**, 422

Cobalamin and the Enzymic Formation of a Factor Concerned in the Synthesis of Methionine by *Escherichia coli*

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The formation of methionine from homocysteine and serine by extracts of acetone-dried *Escherichia coli* PA 15 (a serine- or glycine-requiring auxotroph) is dependent on the presence of some form of folic acid for the transfer of the one-carbon-atom unit and this can be supplied by an extract of the heated organism (Cross & Woods, 1954; Szulmajster & Woods, 1960). (The term folic acid is used in this paper in general reference to the family of compounds; specific members will be referred to by the names in common use, e.g. tetrahydropteroylglutamic acid.) Tetrahydropteroylglutamic acid could not replace this heated extract unless the source of enzyme was organisms which had been harvested from a medium containing cobalamin (Kisliuk & Woods, 1957, 1960). However, enzyme-containing extracts prepared by ultrasonic disruption of the same organism were able to use tetrahydropteroylglutamic acid if free cobalamin was added to the reaction mixture; with an extract of heated *E. coli* as source of folic acid no cobalamin was required (Guest, Helleiner, Cross & Woods, 1960). The synthesis of methionine by ultrasonic extracts of *E. coli* strain 121/176 (a cobalamin or methionine-requiring auxotroph) depended on the presence of cobalamin when either tetrahydropteroylglutamic acid or extract of heated *E. coli* was used as source of folic acid (Helleiner, Kisliuk & Woods, 1957; Guest *et al.* 1960).

A heat-labile and non-diffusible material was partially purified from acetone-dried powders of strain PA 15 cultured in the presence of cobalamin; this material (hereinafter called X) enabled tetrahydropteroylglutamic acid to be used as the co-factor for C_1 transfer in methionine synthesis by extracts of acetone-dried powders of the same organism grown in the absence of cobalamin (Woods, 1958; Kisliuk & Woods, 1960).

It appeared possible that ultrasonic extracts were able to form X in the presence of cobalamin, and that treatment of the organism with acetone destroyed an enzyme or enzymes required for its formation. Indirect evidence for the formation of X by ultrasonic extracts was also obtained by using inhibitory analogues of cobalamin. The action of cobalamin in methionine synthesis was inhibited by the anilide of the monocarboxylic acids of cobalamin, but with extracts of organisms grown in the presence of cobalamin this was not the case (Guest, 1960).

In this paper direct evidence is given for the formation of X by ultrasonic extracts together with some properties of the system. Brief accounts of this work have been published (Guest, 1959; Woods, 1958).

Towards the end of this work, the existence of a cobalamin-containing enzyme, forming part of the complex of enzymes catalysing the transfer of C-3 of serine to homocysteine by a cobalamin-requiring auxotroph of *E. coli*, was reported (Hatch, Takeyama & Buchanan, 1959).

METHODS AND MATERIALS

Extracts of Escherichia coli

Organisms. The strain (PA 15) of *E. coli* used for most of the work was an auxotroph requiring serine or glycine for growth. Strain 121/176 (Davis & Mingioli, 1950) required cobalamin or methionine, whereas strain 3/62 required methionine and did not respond to either cobalamin or homocysteine; the latter strain was isolated from *E. coli* 518 (ATCC 9723) in this Laboratory by the technique of Adelberg & Meyers (1953). All strains were maintained on tryptic meat–agar slopes, subcultured monthly, incubated for 16 hr. at 37° and stored at 4°.

Ultrasonic extracts. These were used mainly as source of enzyme in studies of the formation of X. Their preparation was as described by Guest *et al.* (1960); for strains 121/176 and 3/62 the original growth medium was supplemented with 0·5 mM-DL-methionine.

Extracts of acetone-dried organisms. These were used mainly in the assay of X. Large-scale production of acetone-dried *E. coli* PA 15 was as described by Szulmajster & Woods (1960). For a few experiments the organism was grown in the presence of cobalamin; in this case the vitamin (13 μm-moles) was added with the inoculum to the surface of each tray containing 500 ml. of solidified medium.

The acetone-dried organisms were extracted by mixing with water (10 ml./g. of powder) in a Potter–Elvehjem homogenizer. After centrifuging (25 000 *g*, 45 min.) at 0° the supernatant fluid was dialysed for 18 hr. at 3° against 0·05 M-phosphate buffer, pH 7·8 (750 ml./g. of dried organism); the dialysis fluid was changed once. The product was the extract of acetone-dried organisms normally used. In some experiments the procedure was modified by subjecting the homogenized material to

ultrasonic vibration (25 kcyc./sec., 2·5 min. at 600 w with an ultrasonic generator type E7590B of Mullard Ltd., London) before centrifuging and dialysing. Such extracts were only used with organisms grown without cobalamin.

Extract of heated E. coli. This was prepared as described by Guest *et al.* (1960).

General. Enzymic extracts were sometimes treated with Dowex-1 resin (chloride form, ×8, 100–200 mesh) as described by Kisliuk & Woods (1960). Extracts have been stored at −14° for up to 4 weeks with only moderate loss of activity, though they were normally used immediately after preparation. Their protein content was estimated spectrophotometrically according to Layne (1957).

Formation of the factor

Ultrasonic extracts of organisms were incubated with cobalamin in reaction mixture *A*, which contained (per ml.): glucose, 15 μmoles; adenosine triphosphate (ATP), 7·5 μmoles; $MgCl_2$, 7·5 μmoles; diphosphopyridine nucleotide (DPN), 0·75 μmole; potassium phosphate buffer, pH 7·8, 167 μmoles. The concentration of ultrasonic extract used was equivalent to 7·5 mg. of protein/ml. and the reaction was carried out in a final volume of 3–6 ml. under an atmosphere of H_2 at 37°. In early experiments the concentration of cobalamin was 50 $\mu\mu$moles/ml. and incubation was for 3–4 hr. Later the concentration of the vitamin was increased to 0·5 μm-mole/ml. and the time of incubation decreased to 45 min. The reaction was stopped by cooling the mixture to 0°.

Assay of the factor

During the first part of the work the method used was based on the work of Kisliuk & Woods (1960). A modified method was developed during the present work and will be described in the Results section.

Separation of the factor. Partly purified material, containing the bulk of the X formed during the reaction, was obtained by treatment with protamine sulphate. This reagent (2%; 0·1 ml./ml. of reaction mixture) was stirred in and the mixture stood for 20 min. at 0° before centrifuging at 25 000 *g* for 5 min. The residue (first protamine precipitate, P1) was normally discarded and the supernatant fluid dialysed against 0·05 M-phosphate buffer pH 7·8 for 18 hr. at 3° (150 ml. of buffer/ml. of original reaction mixture); the dialysis fluid was changed twice. During the dialysis a fine white precipitate formed (second protamine precipitate, P2). After centrifuging (15 000 *g*, 10 min.) P2 was suspended in half the original reaction volume of 0·2 M-phosphate buffer, pH 7·8.

Estimation. Determination of the content of X in P2 depended on its ability to promote methionine synthesis by extracts of acetone-dried organisms (cultured without cobalamin) in a system containing tetrahydropteroylglutamic acid as cofactor for the C_1 transfer (Kisliuk & Woods, 1960). The samples used were generally equivalent to 6 mg. of the protein of the ultrasonic extract used to produce X.

The reaction mixture (*B*) used to follow the synthesis of methionine contained (in 2·2 ml. final volume): DL-homocysteine, 10 μmoles; L-serine, 10 μmoles; fructose 1:6-diphosphate, 15 μmoles; ATP, 10 μmoles; $MgSO_4$, 10 μmoles; DPN, 1 μmole; pyridoxal phosphate, 1 μmole; tetrahydropteroylglutamic acid, 1 μmole; potassium phosphate buffer, pH 7·8, 250 μmoles. The enzyme system (dialysed extract of acetone-dried organisms) was added in an amount equivalent to 10 mg. of protein (normal extract) or 20 mg. of protein (ultrasonic-treated extract). Incubation was for 3 hr. at 37° in an atmosphere of H_2. After heating at 100° for 3 min. to stop the reaction, the precipitated protein was removed by centrifuging, and the supernatant fluid assayed for methionine, by the microbiological method of Gibson & Woods (1960), with *Leuconostoc mesenteroides* P60.

In each assay there were controls in which the enzyme was incubated with and without cobalamin in reaction mixture *B* in the absence of a sample containing X; the values for methionine obtained were deducted from the experimental values. Controls in which the samples containing X were incubated in reaction mixture *B* without the extract of acetone-dried organisms were also carried out as a routine.

Expression of results. The activity of the enzymic extract used for assaying X varied slightly from batch to batch and the conditions for the assay were not completely reproducible; it was not possible therefore to define an absolute unit for X. Each complete experiment included a sample of X formed under standard conditions, and this was titrated for its effect on methionine synthesis; the titration of other samples was compared with this. The arbitrary unit of activity of X chosen was that amount which caused the synthesis of 0·1 μmole of L-methionine in a particular assay. In some experiments the results are recorded simply as the total amount of methionine (μm-moles) formed.

Assay of cobalamin

The method described by Kisliuk & Woods (1960) with *Euglena gracilis* var. *bacillaris* was adopted.

Chemicals

The source of cobalamin was 'Anacobin' (British Drug Houses Ltd., Poole, Dorset). The methylamide, ethylamide and anilide derivatives of the monocarboxylic acids of cobalamin (Smith, Parker & Gant, 1956) were kindly provided by Dr E. Lester Smith, who also gave the sample of Factor B (the analogue of cobalamin lacking the nucleotide residue). 5:6-Dimethylbenzimidazolylcobamide-coenzyme was a gift from Dr H. A. Barker; precautions were taken to exclude light in experiments with this compound. The cobalamins were all used as aqueous solutions and were assayed spectrophotometrically by the methods quoted by Guest (1960).

Protamine sulphate (from herring roe) was obtained from L. Light and Co. Ltd., Colnbrook, Bucks., and fructose 1:6-diphosphate from British Drug Houses Ltd., Poole, Dorset. Other chemicals used and their sources have been described by Guest *et al.* (1960).

RESULTS

Trial experiments showed that if ultrasonic extracts were first incubated with cobalamin in the presence of glucose, ATP, Mg^{2+} ions, DPN and phosphate, then the initial rate of synthesis of methionine, on subsequent addition of homocysteine, serine and tetrahydropteroylglutamate, was double that attained when cobalamin was

omitted from the first incubation but was present during the second. This suggested that metabolism of cobalamin led directly or indirectly to the production of material which was more immediately active than cobalamin itself in promoting the utilization of tetrahydropteroylglutamate as cofactor for the C_1 transfer.

Production and properties of the factor

Synthesis by ultrasonic extracts. The assay of X depends upon its ability to promote synthesis of methionine by extracts of acetone-dried organisms when the form of folic acid supplied is tetrahydropteroylglutamate; free cobalamin is not active. In order to obviate the possibility of X being formed during the assay procedure it was first necessary to separate the X formed by the ultrasonic extracts as far as possible both from the enzymes concerned in its formation and from free cobalamin; it was also desirable to remove from the samples other enzymes which participate in the conversion of serine and homocysteine into methionine. This was achieved by treatment of the reaction mixture with protamine sulphate as described in the Methods section.

With relatively small samples of partly purified X (second protamine precipitate, P2) synthesis of methionine in the assay system showed a linear relationship to the size of the sample (Fig. 1), though with larger samples a proportionate increase was not obtained; possibly some other factor or enzyme becomes rate-limiting. A satisfactory assay of X was obtained by using the range over which the response was linear. Samples equivalent to 4·5 or 6 mg. of protein of the original ultrasonic extract were normally used; these caused the synthesis of less than 1 μmole of L-methionine.

The addition of free cobalamin to the assay system had no significant effect (Fig. 1). There was a small but significant formation of methionine in the control in which cobalamin had been omitted from the system for synthesis of X but added to the assay system (Fig. 1). The validity of the assay was not affected by this since free cobalamin in the samples would have been largely removed by dialysis in the course of their preparation. It is probable that the treatment with protamine did not completely remove the enzymes concerned in the formation of X and that this proceeds slowly during the assay procedure if sufficient free cobalamin is present. The partly purified samples of X did not cause the synthesis of any methionine in the assay system if the enzyme preparation (extract of acetone-dried organisms) was omitted. There was no synthesis of X during its separation from the reaction mixture. This was shown by adding cobalamin immediately after incubation to a control reaction mixture not containing the vitamin and isolating the P2 fraction; it had less than 5% of the activity of a similar fraction isolated after incubation with cobalamin.

The first protamine precipitate (P1, see Methods section) and the supernatant fluid remaining after removing P2 had no activity, nor did they affect the activity of P2. Furthermore, the activity of P2 was apparently the same as that of an equivalent quantity of the reaction mixture which had simply been dialysed. It is probable therefore that all of the X is precipitated with the P2 fraction. When the suspension of P2 in 0·2 M-potassium phosphate buffer pH 7·8 was centrifuged, all the activity was recovered in the supernatant fluid. An overall purification of X of four- to five-fold was achieved during its separation; this was shown by dry-weight determinations after exhaustive dialysis of the fractions against water.

It is concluded, from the above experiments, that incubation of cobalamin with ultrasonic extract, in a suitable reaction mixture, leads to the production of material with similar biological activity to that extracted by Kisliuk & Woods (1960) from acetone powders of organisms grown in the presence of cobalamin.

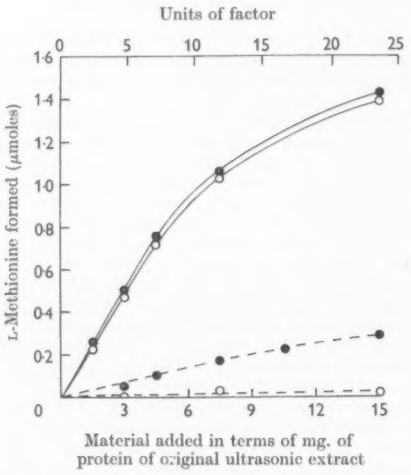

Fig. 1. Formation of the factor (X) by ultrasonic extracts. Reaction mixture *A*, with cobalamin (50 $\mu\mu$moles/ml.) present (—) or absent (- - -), was incubated for 4 hr. The reaction mixtures were then treated to obtain the second protamine precipitates (P2) and the latter assayed for 'X' by using reaction mixture *B* with normal extract of acetone-dried organisms equivalent to 10 mg. of protein and with cobalamin (65 $\mu\mu$moles) present (●) or absent (○); incubated 3 hr. Units of factor activity were deduced from the curve ○—○.

Other properties of the factor. These tests were carried out with the partly purified P2 fraction. The cobalamin content as measured with *Euglena gracilis* was 7% of the total cobalamin added to the original reaction mixture. For a sample of the size used for assay this corresponds to only about 2 $\mu\mu$moles of cobalamin compared to the 65 $\mu\mu$moles added to the assay system in the controls of the experiments of Fig. 1. It is not known whether the cobalamin in fraction P2 is free or bound; the fraction had, however, been extensively dialysed.

The activity of X was not decreased by dialysis for 48 hr. against 0·05 M-potassium phosphate buffer pH 7·8. It was heat-labile; 70% of the activity was lost after 5 min. at 50° and 100% after 5 min. at 60°. The preparations of X were unstable at pH values below 5 and above 8·5; for example, half the activity was lost in 30 min. at 0° at either pH 4 or 9·8. These properties are again in accord with those of the material separated from cobalamin-grown organisms by Kisliuk & Woods (1960) and suggest that X is a protein or at least closely associated with protein.

Effect of analogues of cobalamin

Certain analogues of cobalamin inhibit the action of cobalamin in promoting methionine synthesis by ultrasonic extracts when tetrahydropteroylglutamate is the cofactor for the C_1 transfer. However the anilide analogue does not inhibit methionine synthesis if the enzymic extracts are prepared from organisms grown with cobalamin, though synthesis was reduced if the analogue as well as cobalamin was added to the growth medium (Guest, 1960). These results suggested that the analogues may interfere with the formation of X, though not with its activity once formed.

Effect of analogues of cobalamin on the activity of the factor. The anilide of the monocarboxylic acids of cobalamin had no effect on the activity of the partly purified preparation of X derived from the incubation of cobalamin with ultrasonic extract (Table 1). A more extended series of tests was carried out with a crude preparation of X derived from acetone-dried organisms grown in the presence of cobalamin (Kisliuk & Woods, 1960). The amount of the preparation used itself catalysed the formation of negligible quantities of methionine and contained less than 15 $\mu\mu$moles of cobalamin. The anilide analogue again had no effect on the activity of the X (Table 1); this was also the case with the methylamide and ethylamide analogues. Factor B (the analogue of cobalamin containing no nucleotide residue) did however inhibit about 40% at relatively high concentrations; the inhibition (by 70 μm-moles) was not relieved by cobalamin (6·5 μm-moles). The inactivity of the anilide analogue was not due to its destruction in the test system. Furthermore, in controls in which X was omitted the analogues did not themselves promote methionine synthesis in the assay system.

Effect of analogues of cobalamin on the formation of the factor. All the analogues tested inhibited the formation of X completely if sufficiently high concentrations were used (Table 2). The ratios of concentration of cobalamin to analogue resulting in 50% inhibition were similar to those reported by Guest (1960) for the inhibition of the activity of cobalamin in methionine synthesis. The analogues themselves, when incubated with ultrasonic extracts, did not give rise to significant X activity (Table 2). Only in the case of Factor B was it possible that a carry-over of the test substance into the final assay system could vitiate the results by inhibiting the function of X itself (see last paragraph): it is unlikely, however, that inhibitory concentrations would remain after the treatment, with protamine sulphate and dialysis, used in preparing the samples for assay.

The conclusion that the anilide analogue inhibited the formation of X, though not its subsequent activity, was supported by the experiment of Table 3. Cobalamin was required for the synthesis of methionine by ultrasonic extracts with tetrahydropteroylglutamate as the cofactor for the C_1 transfer, and such synthesis was completely inhibited by the anilide analogue. A preparation of X replaced cobalamin, but in this case the anilide analogue was not inhibitory. For this experiment a more purified preparation of X derived from acetone-dried organisms grown with cobalamin was used (M. A. Foster & K. M. Jones, unpublished work in this Laboratory); it had a higher ratio of

Table 1. *Effect of analogues of cobalamin on the activity of the factor*

Source of factor. (I) Second protamine precipitate, P2, after incubation of cobalamin (50 $\mu\mu$moles/ml.) with sonic extract in reaction mixture *A* for 4 hr.; sample used was equivalent to 6 mg. of protein of the sonic extract. (II) Dialysed extract (equivalent to 2 mg. of protein) of acetone-dried organisms grown in the presence of cobalamin.

Assay of activity of the factor. Reaction mixture *B* plus (I) normal acetone-powder extract (10 mg. of protein) or (II) ultrasonic-treated acetone-powder extract (20 mg. of protein). Incubated 3 hr.

Source of factor	Analogue (μm-moles)	L-Methionine formed (μm-moles)
I	None	1050
	Anilide (5·7)	1070
	(57·0)	1070
II	None	575
	Anilide (1·2)	580
	(57·0)	590
	Factor B (3·5)	465
	(70·0)	345

specific activity to cobalamin content than the usual P 2 preparation so that even less free cobalamin was added with the sample of X.

No evidence could be obtained for the production of an inhibitory analogue of X when the anilide analogue was incubated with ultrasonic extract under the same conditions as led to the production of X from cobalamin. A second protamine precipitate fraction was isolated from this reaction mixture; when added to the assay system at five times the concentration of a similar preparation of X it did not inhibit the methionine production caused by the latter.

Table 2. *Effect of cobalamin analogues on the synthesis of the factor*

Synthesis of factor. Reaction mixture *A* plus cobalamin (50 $\mu\mu$moles/ml.) when present and analogues as stated. Incubated 3·5 hr. Samples assayed were the P2 fractions (see text) and were equivalent to 6·0 mg. of original ultrasonic protein.

Assay of factor. Reaction mixture *B* plus normal extract of acetone-dried organisms (10 mg. of protein). Incubated 3 hr.

Analogue (μm-moles/ml.)	Units of factor formed with cobalamin: Absent	Present
None	0·5	12·0
Methylamide (4·4)	2·4	3·0
(44·0)	2·2	2·1
Ethylamide (1·3)	—	5·7
(43·0)	1·3	1·8
Anilide (0·8)	—	3·6
(42·0)	1·3	1·3
Factor B (2·6)	—	4·4
(51·0)	0·7	0·8

Table 3 *Effect of the anilide analogue of cobalamin on the synthesis of methionine by ultrasonic extracts*

Reaction mixture for the synthesis of methionine (Guest, 1960) plus tetrahydropteroylglutamate (1 μmole) and ultrasonic extract of *E. coli* (equivalent to 10 mg. of protein). Cobalamin (65 $\mu\mu$moles) and the anilide analogue (30 μm-moles) when present. Purified preparation of factor X (see text) equivalent to 0·1 mg. of protein. Incubated 4 hr.

Additions	L-Methionine formed (μm-moles/mg. of protein) with anilide analogue: Absent	Present
None	4	10
Cobalamin	70	10
Factor X	38	40
Cobalamin + factor X	110	42
Factor X (no ultrasonic extract)	2	—

Use of analogues of cobalamin in the assay of the factor

The results of the preceding section suggested a more convenient method for the assay of X in which it would not be necessary to separate it (as the P 2 fraction) from the reaction products. Addition of the anilide analogue to the reaction mixture (*B*) for the assay should prevent the formation of X during the assay itself from cobalamin carried over with the sample, provided that the concentration of the cobalamin is not too great; dialysis alone should reduce the concentration sufficiently.

Ultrasonic extract was incubated with cobalamin in the usual reaction mixture. One portion of the mixture after incubation was simply dialysed against 0·05 M-potassium phosphate buffer pH 7·8 for 18 hr. at 3° (150 ml. of buffer/ml. of reaction mixture); the dialysis fluid was changed twice. The other portion was treated with protamine and a P 2 fraction obtained as in Methods. Both types of sample were assayed for X activity in the usual test system (see Methods) but with the anilide analogue either present or absent. Two special controls were done. In one, cobalamin was omitted from the reaction mixture for synthesis of the factor, and, in the other, cobalamin at a relatively high concentration (30 $\mu\mu$moles/ml.) was added to the reaction mixture for assay. The activities of the two types of sample were similar (Table 4) whether the anilide analogue was present or not in the assay system. However, slight doubt was cast on the validity of the assay (with anilide absent) on the sample which had been dialysed only, because the control in which cobalamin had been absent from the synthesis system but present in the assay system gave a higher value than a similar control for the P 2 fraction. This apparent X activity was greatly decreased by the addition of the anilide analogue to the assay system (Table 4).

The main disadvantage of this method for assay of X was that when the normal enzyme preparation (extract of acetone-dried organisms) was omitted from the assay system there was a small synthesis of methionine; this can be attributed to the enzymes of the ultrasonic extract carried over with the sample. Under the conditions normally used this was equivalent to 2–3 units of X. The difficulty was overcome (*a*) by ensuring that all systems for assay of X contained the same amounts of protein of similar origin and (*b*) by increasing the amount of the main enzyme source (extract of acetone-dried organisms) so that its concentration could not be rate-limiting.

The final procedure for this alternative assay of X (which was adopted for most of the work still to be described) was to dialyse samples against

Table 4. *The anilide analogue of cobalamin and the assay of the factor*

Synthesis of factor. Reaction mixture *A* with cobalamin (50 μμmoles/ml.) when present. Incubated 4 hr. Samples of factor tested (each equivalent to 6 mg. of protein of the original ultrasonic extract): (I) Second protamine precipitate (P2) fraction from reaction mixture, (II) reaction mixture dialysed only.

Assay of factor. Reaction mixture *B* with cobalamin and anilide analogue as shown; normal extract of acetone-dried organisms equivalent to 10 mg. of protein. Incubated 3 hr.

			Assay of the factor		
			Units of factor activity in the sample in presence of anilide analogue (μm-moles)		
Sample	Cobalamin (during synthesis of factor)	Cobalamin (65 μμmoles)	0	5·7	57·0
(I) (P2 fraction)	Present	Absent	10·8	11·0	11·0
		Present	11·2	—	—
	Absent	Absent	0·2	—	—
		Present	1·8	0·5	0·2
(II) (Dialysed only)	Present	Absent	11·5	—	10·8
		Present	11·3	—	—
	Absent	Absent	0·4	—	0·8
		Present	3·2	1·5	0·9

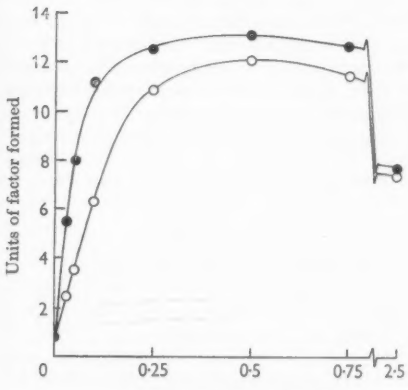

Fig. 2. Effect of cobalamin concentration on the synthesis of the factor by ultrasonic extracts. Synthesis of factor: reaction mixture *A* plus Dowex-1-treated ultrasonic extract and cobalamin; incubated 1 hr. (○) or 3 hr. (●). Dialysed samples equivalent to 6 mg. of protein of the original ultrasonic extract were used for assay. Assay of factor: reaction mixture *B* plus extract of acetone-dried organisms (20 mg. of protein) and anilide analogue (46 μm-moles); incubated 3 hr.

0·05 M-potassium phosphate buffer pH 7·8, and to assay them in the reaction mixture *B* in the presence of the anilide analogue (23 or 46 μm-moles) and with the source of enzyme (extract of acetone-dried organisms) increased to the equivalent of 20 mg. of protein. The curve relating size of sample to amount of methionine synthesized was similar to that of Fig. 1 for the original method of assay, except that the linear range was slightly reduced. Addition of cobalamin to the assay system did not increase the methionine produced by either experimental or control samples of the reaction mixture for the synthesis of X.

The new method of assay for X enabled its formation to be studied when changes were made in the synthesis system, either by purification of the ultrasonic extract or by altering the composition of reaction mixture *A*, which made protamine treatment no longer practicable.

Properties of the system forming the factor

Effect of the concentration of cobalamin. An optimum synthesis of X during the first hour of incubation was obtained with 0·5 μm-mole of cobalamin/ml. (Fig. 2); synthesis was proportional to time up to 45 min. With higher concentrations of cobalamin, although the initial rate was increased, synthesis of X stopped after a short period; the reason for this is not known. In subsequent experiments a cobalamin concentration of 0·5 μm-mole/ml. and 45 min. incubation was used as a routine and 46 μm-moles of the anilide analogue was added to the assay system. The cobalamin content of the resultant dialysed samples, used for assay of X, was estimated by assay with *Euglena gracilis* as 0·1 μm-mole.

5:6 - *Dimethylbenzimidazolylcobamide - coenzyme.* This is one of several coenzyme forms of the cobalamins which functions in the formation of β-methylaspartate from glutamate by *Clostridium tetanomorphum* (Weissbach, Toohey & Barker, 1959; Barker *et al.* 1960). It is 2–3 times as active as cobalamin in the synthesis of methionine by ultrasonic extracts of *E. coli* and is also less sensitive to inhibition by the anilide analogue of cobalamin (Guest *et al.* 1960; Guest, 1960).

The coenzyme did not replace X for its activity in the present test system nor did it affect the activity of X. It did however replace cobalamin for the formation of material with the activity of X (Table 5). Such synthesis was inhibited 80 % by a concentration of the anilide analogue which completely prevented the synthesis of X in the presence of cobalamin. Other requirements for the formation of X from the cobamide-coenzyme were similar to those for cobalamin (Table 6).

Other requirements for the synthesis of the factor. Omission of any of the constituents of reaction mixture *A* decreased the rate of formation of X, the effect of ATP + Mg^{2+} ions and of glucose being the most pronounced (Table 6). Absence of DPN decreased synthesis by about 40 %. Reduced DPN however replaced both DPN and glucose, and at higher concentrations also eliminated the requirement for ATP (Table 6). Addition of the substrates and the cofactor for the synthesis of methionine itself (homocysteine, serine and tetrahydropteroylglutamate) had no significant effect on the formation of X.

The presence of substances possibly required for protein synthesis (mixtures of amino acids, and mixtures of amino acids, purines, pyrimidines and B-group vitamins) did not increase synthesis of X, nor was such synthesis inhibited by *p*-fluorophenylalanine (mM), 4-methyltryptophan (mM) and 8-azaguanine (0·2 mM). Chloramphenicol (0·14 mM) inhibited by 30 % only; it could not be used at higher concentrations since it then inhibited the growth of the organism used for the assay of methionine.

Activity of the ultrasonic extract in synthesizing X. The synthesis of X increased linearly with the concentration of ultrasonic extract up to the limit tested (11 mg. of protein/ml.). Different batches of the extract varied in activity by an overall factor of two, but there was generally no loss of activity on storage for up to 6 weeks at −14°. The extract lost 90 % of its activity on heating to 45° for 3 min. Treatment with Dowex-1 resin caused only a 10–15 % decrease in activity which could be restored by the addition of the heated extract of organisms.

Ultrasonic extracts of *E. coli* strain 121/176 and 3/62 also catalysed the synthesis of X, though the activity of strain 3/62 was only 10–20 % of that of strains PA 15 and 121/176.

Table 5. 5:6-*Dimethylbenzimidazolylcobamide-coenzyme and the synthesis of the factor by ultrasonic extracts*

Synthesis of factor. Reaction mixture *A*, cobalamin or 5:6-dimethylbenzimidazolylcobamide-coenzyme (each 50 μμmoles/ml.) and anilide analogue (95 μm-moles/ml.) when added. Incubated 4 hr. Samples assayed were P2 fractions equivalent to 6 mg. of protein of the ultrasonic extract.

Assay of factor. Reaction mixture *B* plus ultrasonic-treated extract of acetone-dried organisms (20 mg. of protein) and anilide analogue (23 μm-moles). Incubated 3 hr.

Additions	Units of factor formed
None	0·7
Cobalamin	10·0
Cobalamin + anilide analogue	0·9
Anilide analogue	0·9
Cobamide-coenzyme	11·2
Cobamide-coenzyme + anilide analogue	3·0

DISCUSSION

The difference in behaviour of extracts of acetone-dried organisms and ultrasonic extracts appears to lie in the ability of the latter to utilize free cobalamin in the formation of a factor (X), which is required for the synthesis of methionine by cell-free extracts of *E. coli* PA 15 when tetrahydropteroylglutamate is provided as the cofactor for C_1 transfer. Treatment with acetone appears almost completely to destroy the capacity of the organism

Table 6. *Requirements for the formation of the factor by ultrasonic extracts of* E. coli PA 15

Synthesis of factor. Reaction mixture *A* plus ultrasonic extract (treated with Dowex-1; see Methods) and cobalamin (0·5 μm-mole/ml.) or 5:6-dimethylbenzimidazolylcobamide-coenzyme (0·4 μm-mole/ml.). Incubated 45 min. Samples for assay were equivalent to 4·5 mg. of protein of the ultrasonic extract.

Assay of factor. Reaction mixture *B* plus ultrasonic-treated extract of acetone-dried organisms (20 mg. of protein) and anilide analogue (46 μm-moles). Incubated 3 hr. $DPNH_2$, reduced diphosphopyridine nucleotide.

Omissions from reaction mixture *A*	Additions	Units of factor formed with: Cobalamin	Units of factor formed with: Cobamide-coenzyme
None	—	9·5	11·0
Cobalamin or cobamide-coenzyme	—	0·6	0·9
Glucose	—	2·2	4·0
DPN	—	6·0	5·0
ATP and Mg^{2+} ions	—	2·4	3·2
Glucose and DPN	$DPNH_2$ (4 μmoles)	9·5	—
Glucose, DPN and ATP	$DPNH_2$ (16 μmoles)	9·0	—

to form X, though acetone does not affect X once formed. Extracts of such acetone-dried organisms cannot synthesize methionine with tetrahydropteroylglutamate as source of folic acid and do so only very feebly on the addition of free cobalamin. Extracts of acetone-dried organisms may therefore be used for the assay of the X which is formed either by growing the organisms in the presence of cobalamin or, as in the present work, incubating cobalamin with ultrasonic extract in the presence of glucose, ATP and DPN.

The properties of the factor formed in the two different ways are closely similar and suggest that it is either a protein or firmly associated with a protein. Although the material contains cobalamin it cannot be said at the present low degree of purification whether this is adventitious or whether X contains a cobalamin residue. The fact that one analogue of cobalamin (Factor B) at relatively high concentrations partially inhibits the activity of X provides some evidence that the latter may have a cobalamin residue at its active centre; other analogues of cobalamin do not, however, inhibit. All the analogues inhibited the formation of X; this could be interpreted either as inhibition of the incorporation of a cobalamin residue into X or as inhibition of a catalytic function of cobalamin in the synthesis of X.

The formation of X requires reducing power and possibly a source of energy. Although *de novo* synthesis of protein cannot be excluded since there was slight inhibition by chloramphenicol it is more likely that some pre-existing protein is modified; synthesis was not increased by mixtures of amino acids, purines and pyrimidines and was not inhibited by analogues of these substances which would be expected to inhibit protein synthesis.

5:6-Dimethylbenzimidazolylcobamide-coenzyme replaced cobalamin for the synthesis of X and may be more active. However no direct evidence was obtained for or against the cobamide-coenzyme being an intermediate in the function of cobalamin in the formation of X.

If X is indeed a protein the question arises as to whether it has enzymic activity in some reaction or whether it has catalytic activity in some other sense, perhaps analogous to the function of cytochrome. No direct enzymic action of X on, for example, tetrahydropteroylglutamate has so far been detected. The function of X in methionine synthesis, and particularly its relationship to the utilization of tetrahydropteroylglutamate, is not yet known; various hypotheses have been discussed previously (Kisliuk & Woods, 1960; Guest *et al.* 1960; Guest, 1960). Hatch *et al.* (1959) have shown a cobalamin-containing enzyme to be part of the complex of enzymes catalysing the transfer of the β-carbon atom of serine to homocysteine by a cobalamin auxotroph of *E. coli*; tetrahydropteroylglutamate was the source of folic acid in this system also.

Ultrasonic extracts of a strain (121/176) of *E. coli* requiring cobalamin or methionine for growth also form X when incubated with cobalamin. Extracts of this organism require cobalamin for methionine synthesis even when folic acid is supplied by an extract of heated *E. coli* (Guest *et al.* 1960); it is likely therefore that the function of X in methionine synthesis is not solely concerned with the utilization of tetrahydropteroylglutamate.

E. coli strain 3/62 has an absolute requirement for methionine for growth, and cell-free extracts have no ability to synthesize it. Nevertheless ultrasonic extracts do form X, though in less quantity than the other strains. This emphasizes that concurrent synthesis of methionine is not necessary or helpful to the formation of X; it will be recalled that the addition of homocysteine, serine and tetrahydropteroylglutamate had no effect on the formation of X by extracts of strain PA 15.

SUMMARY

1. A heat-labile, non-diffusible material has been partly purified from the products of the incubation of ultrasonic extracts of *Escherichia coli* strain PA 15 with cobalamin, glucose, ATP, Mg^{2+} ions, DPN and phosphate buffer. This factor is essential for the synthesis of methionine by extracts of acetone-dried organisms of the same strain when tetrahydropteroylglutamate acts as source of the necessary folic acid cofactor for the C_1 transfer reaction.

2. The factor has similar properties to material with the same biological activity previously isolated from the same organism grown in the presence of added cobalamin.

3. Monosubstituted cobalamin-amide analogues inhibit the formation of the factor by the ultrasonic extracts but have no effect upon its function in methionine synthesis. Factor B partially inhibits both the formation and the function of the factor.

4. A method for assaying the factor formed by ultrasonic extracts is described. It makes use of the ability of analogues of cobalamin to inhibit formation of the factor.

5. 5:6-Dimethylbenzimidazolylcobamide-coenzyme has no activity as the factor but replaces cobalamin for its formation.

One of us (J.R.G.) is indebted to the Medical Research Council for a Training Scholarship. Our thanks are due to Dr E. Lester Smith and Dr H. A. Barker for providing analogues and derivatives of cobalamin. We are grateful to the Director and staff of the Microbiological Research Establishment, Porton Down, Wilts, for undertaking the large-scale growth of organisms. The research was aided by a grant for expenses from The Nuffield Foundation.

REFERENCES

Adelberg, E. A. & Meyers, J. W. (1953). *J. Bact.* **65**, 348.
Barker, H. A., Smyth, R. D., Weissbach, H., Toohey, J. I., Ladd, J. N. & Volcani, B. E. (1960). *J. biol. Chem.* **235**, 480.
Cross, M. J. & Woods, D. D. (1954). *Biochem. J.* **58**, xvi.
Davis, B. D. & Mingioli, E. S. (1950). *J. Bact.* **60**, 17.
Gibson, F. & Woods, D. D. (1960). *Biochem. J.* **74**, 160.
Guest, J. R. (1959). *Biochem. J.* **72**, 5P.
Guest, J. R. (1960). *Biochem. J.* **76**, 405.
Guest, J. R., Helleiner, C. W., Cross, M. J. & Woods, D. D. (1960). *Biochem. J.* **76**, 396.
Hatch, F. T., Takeyama, S. & Buchanan, J. M. (1959). *Fed. Proc.* **18**, 243.
Helleiner, C. W., Kisliuk, R. L. & Woods, D. D. (1957). *J. gen. Microbiol.* **18**, xv.
Kisliuk, R. L. & Woods, D. D. (1957). *J. gen. Microbiol.* **18**, xv.
Kisliuk, R. L. & Woods, D. D. (1960). *Biochem. J.* **75**, 467.
Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 451. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Smith, E. L., Parker, L. F. J. & Gant, D. E. (1956). *Biochem. J.* **62**, 14P.
Szulmajster, J. & Woods, D. D. (1960). *Biochem. J.* **75**, 3.
Weissbach, H., Toohey, J. & Barker, H. A. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 521.
Woods, D. D. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **11**, 302.

Biochem. J. (1960) **77**, 430

The Metabolism of C_2 Compounds in Micro-organisms

4. SYNTHESIS OF CELL MATERIALS FROM ACETATE BY *ASPERGILLUS NIGER**

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Many fungi grow readily on C_2 compounds as the sole source of carbon and excrete, into their growth media, intermediates of the tricarboxylic acid cycle or compounds derived therefrom. The tricarboxylic acid cycle has been shown to be the major route both for the catabolism of C_2 compounds such as acetate or ethanol and for the provision of the carbon skeletons of most cellular constituents and of compounds excreted (for review, see Chain, 1955; Foster, 1958; Kornberg, 1959). Its continued operation under these conditions necessitates the occurrence of reactions, ancillary to the tricarboxylic acid cycle, which lead to the net formation of intermediates of that cycle from the C_2 growth substrate.

The main purpose of this paper is to show that *Aspergillus niger* growing on acetate as sole carbon source incorporates isotope from [2-^{14}C]acetate in a manner which excludes the primary formation of succinate from acetate (Thunberg, 1920) and which indicates that the required syntheses of intermediates of the tricarboxylic acid cycle are effected via the glyoxylate cycle (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957). Cell-free extracts contain a high activity of the requisite enzymes of the glyoxylate cycle and can catalyse the net formation of malate and succinate from acetyl-coenzyme A and *iso*citrate. Extracts of *A. niger*, grown on substrates the metabolism of which does not necessitate such syntheses of C_4 compounds from acetate, are virtually devoid of a key enzyme of the glyoxylate cycle, *iso*citratase: this supports the view that that cycle plays an essential role in growth on acetate.

This work has been presented in part to the Biochemical Society (Kornberg & Collins, 1958).

METHODS

Maintenance and growth of the mould. The strain of *A. niger* studied had a high ability for producing citric acid. The organism was maintained on slopes consisting of 50 mM-ammonium acetate, 20 mM-sodium–potassium phosphate buffer, pH 7·4 (prepared by mixing 80·4 vol. of 20 mM-Na_2HPO_4 with 19·6 vol. of 20 mM-K_2HPO_4), salts (25 mg. of $MgSO_4,7H_2O$, 0·1 mg. of $FeSO_4,7H_2O$, 0·1 mg. of $ZnSO_4,7H_2O$, 10 μg. of $MnSO_4,4H_2O$, 1 μg. of $CuSO_4,5H_2O$ and 0·2 μg. of $K_2Cr_2O_7$/100 ml. of medium), 0·05% (w/v) of 'Difco' yeast extract and 2% (w/v) of agar (Hopkin and Williams Ltd., Chadwell Heath, Essex). Stock cultures were grown at 30° until sporulation had taken place (4–7 days), and were then stored at 2°. For growth in liquid medium, a suspension of spores in sterile water was used to inoculate Carrel culture flasks (J. A. Jobling and Co. Ltd., Sunderland) containing 400 ml. of medium which, with the omission of the agar, was identical with that used for the slopes. The flasks were shaken in air at 30° for 20–

* Part 3: Kornberg & Madsen (1958).

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24 hr. Larger quantities of cells were grown after inoculation of 9 l. of medium, contained in an aspirator bottle (10 l.) fitted with a sterile air supply to maintain aeration and agitation, at 30°. For growth on glucose, the liquid medium contained the same buffer and salts as before but the ammonium acetate was replaced by 50 mM-glucose and 50 mM-ammonium chloride.

Incorporation of [2-^{14}C]*acetate by whole cells. A. niger* (700 mg. wet wt.) was harvested by filtration after 20 hr. growth on acetate, washed with water and suspended in 60 ml. of 20 mM-potassium phosphate buffer, pH 7·1. One-fifth of this suspension was shaken at 30° in a conical flask, together with 0·4 ml. of 0·5M-ammonium acetate. The flask was shaken for 10 min. at 30°. Sodium [2-^{14}C]acetate (44·4 μmoles, 200 μc) in 1 ml. of water was mixed with 2 ml. of 20 mM-phosphate buffer pH 7·1; 0·25 ml. of this mixture was added to 3 ml. of hot ethanol together with 1 ml. of the *A. niger* suspension. This constituted the first (zero-time) sample. The remainder of the [2-^{14}C]acetate solution was added to the mould suspension, and samples (1 ml.) were taken at measured intervals and pipetted into tubes containing 3 ml. of hot ethanol, standing in a bath at 80°. The subsequent treatment of these samples, extraction with aqueous ethanol, chromatographic analysis, radioautography, assay and identification of the labelled compounds, was as described by Kornberg (1958).

Preparation of extracts from crushed Aspergillus niger. *A. niger* was harvested by filtration after 24 hr. growth, and was washed with 0·9% (w/v) potassium chloride solution. The damp mould was frozen and crushed at −25° in a Hughes press (Hughes, 1951). Extracts were prepared by stirring some crushed mycelia with 4 vol. of 0·1M-potassium phosphate buffer, pH 7·2, at 0°. Some of the suspension was dried overnight at 100° for determination of the dry weight of mould present. The rest was centrifuged for 10 min. at approximately 20 000 *g* in an International refrigerated centrifuge at 0°. The supernatant solution was used in experiments involving enzymic assays.

Incorporation of [^{14}C]*acetate by cell extracts.* The incubation mixtures used for these experiments contained 100 μmoles of potassium phosphate buffer, pH 7·4, 10 μmoles of reduced glutathione, 5 μmoles of magnesium chloride, 0·08 μmole of coenzyme A, 1·9 μmoles of potassium [^{14}C]acetate (containing 10 μc of isotope), 10 μmoles of other unlabelled substrates as indicated, 0·2 ml. of *A. niger* extract and water to 0·95 ml. After incubation for 3 min. at 30°, 5 μmoles of adenosine triphosphate (ATP) tetrasodium salt were added. The reaction was stopped by adding 3 ml. of ethanol; the resulting aqueous ethanolic solutions were analysed as previously described (Kornberg, 1958; Kornberg & Madsen, 1958).

Incorporation of [1-^{14}C]*glyoxylate by cell extracts.* The incubation procedure was similar to that described for incorporation experiments involving [^{14}C]acetate, but the acetate was replaced by 7·5 μmoles of sodium [1-^{14}C]glyoxylate (containing 4·5 μc of isotope), which had been prepared from the calcium salt by treatment with Dowex-50 (Na^+ form).

Enzyme assays. Acetic thiokinase was determined as described by Jones & Lipmann (1955). *iso*Citratase was measured by the procedure of Olson (1959). *iso*Citrate was assayed with the *iso*citric dehydrogenase of *Pseudomonas* KB1 (Kornberg & Madsen, 1958) by spectrophotometric measurement of the reduction of triphosphopyridine nucleotide at 340 mμ (Kornberg, 1955). Succinate was estimated manometrically with the succinoxidase of washed pigeon-breast muscle (Krebs, 1937; Umbreit, Burris & Stauffer, 1957).

Identification of glyoxylate. Glyoxylate semicarbazone, produced by enzymic cleavage of *iso*citrate and interaction of the formed glyoxylate with semicarbazide (Olson, 1959), was decomposed with 6N-hydrochloric acid and a slight excess of saturated 2:4-dinitrophenylhydrazine hydrochloride at room temperature overnight. The 2:4-dinitrophenylhydrazone was extracted into ethyl acetate and this non-aqueous layer was extracted with M-sodium carbonate. This solution was acidified with 6N-hydrochloric acid and re-extracted with ethyl acetate. The quantity of keto acid present in a portion of this solution was estimated by the method of Friedemann & Haugen (1943); the ratio of the extinction at 445 mμ to that at 520 mμ was 2:1, indicating that glyoxylate 2:4-dinitrophenylhydrazone was the sole derivative present. This was confirmed by comparison of the spectra of the enzymically formed material and that of authentic glyoxylate 2:4-dinitrophenylhydrazone. The remainder of the material extracted into ethyl acetate was analysed by ascending chromatography in the solvent of El Hawary & Thompson (1953): again, the product behaved identically with that of authentic glyoxylate 2:4-dinitrophenylhydrazone.

Materials used. Potassium dihydrogen L$_s$-*iso*citrate was a gift from Dr H. B. Vickery and highly purified sodium glyoxylate from Dr I. Zelitch (both at the Connecticut Agricultural Experiment Station, New Haven, Conn., U.S.A.). Pyridine nucleotides and coenzyme A were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.; ATP (tetrasodium salt) from the Zellstoff-Fabrik Waldhof, Wiesbaden, Germany; glutathione from The Distillers Co., Speke, Lancs.; and isotopic materials from The Radiochemical Centre, Amersham, Bucks. All other reagents used were of A.R. grade or of the highest purity commercially available.

RESULTS

Incorporation of [2-^{14}C]*acetate by* Aspergillus niger. When [2-^{14}C]acetate was added to suspensions of *A. niger*, shaken at 30° in acetate growth medium, isotope was rapidly incorporated into cellular constituents. The incorporation into the fraction of the cells soluble in aqueous ethanol was approximately linear with time over the first 4 min. of the experiment (Fig. 1).

Distribution of ^{14}C *amongst the labelled constituents of the soluble fraction.* Two-dimensional chromatography and radioautography of the aqueous ethanol-soluble fractions obtained from each sample of the incubated suspension showed that ^{14}C from acetate rapidly appeared in many of the intermediates of the tricarboxylic acid cycle and in amino acids derived therefrom: even after the shortest time of incubation (13 sec.) isotope was found in malate, citrate, aspartate, glutamate and succinate. Isotope was detected in alanine after 50 sec., but in phosphorylated materials only after more than 5 min. This latter finding showed that

carbon dioxide-fixing reactions, such as operate in photosynthetic (Calvin, 1955) or autotrophic (Santer & Vishniac, 1955; Trudinger, 1955, 1956; Aubert, Milhaud & Millet, 1956, 1957; Kornberg, Collins & Bigley, 1960*a*) organisms were not of primary importance in the incorporation of acetate.

The difficulties of accurately and speedily obtaining samples of uniform size from the heterogeneous suspension of *Aspergillus* were such that only approximate estimates can be made of the rate at which the added [2-^{14}C]acetate was utilized; moreover, materials which are volatile (such as carbon dioxide) or are lost in the chromatographic procedure (such as pyruvate or oxaloacetate) were not assayed. However, the relative distribution of isotope within each sample fairly reflects the time course of incorporation of isotope from acetate.

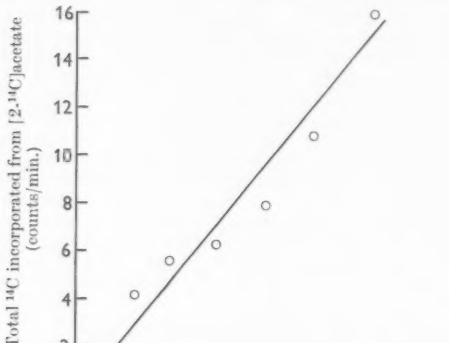

Fig. 1. Incorporation of ^{14}C from [2-^{14}C]acetate into the fraction soluble in aqueous ethanol of *Aspergillus niger* growing on acetate. For conditions, see Methods section.

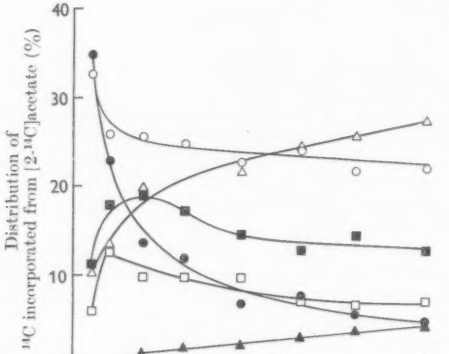

Fig. 2. Variation with time of the percentage distribution of ^{14}C from [2-^{14}C]acetate incorporated into the constituents of the fraction, soluble in aqueous ethanol, of *Aspergillus niger* growing on acetate. For conditions, see Methods section. ●, Citrate; ○, malate; □, succinate; ■, aspartate; △, glutamate; ▲, alanine.

When the proportion of the total radioactivity of each sample contributed by each labelled compound, expressed as the percentage of the total isotope in the sample, was plotted against time, smooth curves could be drawn through the points obtained (Fig. 2). Initially, approx. 35% of the incorporated ^{14}C was present in citrate, and approx. 33% in malate, whereas aspartate and glutamate contained respectively 11 and 10%. Some 5% of the isotope was present in succinate. These proportions rapidly altered with time: both citrate and malate decreased sharply with time, whereas succinate and aspartate, after an initial rise, reached a steady level at which they contributed respectively 9 and 15% of the total radioactivity of each sample.The proportion of isotope present in glutamate rose steadily throughout the incubation, as did that in alanine. After 100 sec., a 'steady state' had been reached: the labelling of the intermediates of the tricarboxylic acid cycle at and after this time may be taken as a reflexion of the 'pool sizes' of these compounds, since, by this time, these intermediates appear to be saturated with the isotope entering from the administered acetate.

The rapid initial decrease with time in the proportion of isotope contained in citrate and the concomitant rise of that in succinate indicates, as expected from the tricarboxylic acid cycle, that isotope from acetate entered citrate before it appeared in succinate. But the behaviour of malate, which is similar to that of citrate, is at variance with that expected from the operation of the tricarboxylic acid cycle; at the earliest times, malate contained nearly 6 times as much isotope as did succinate, whereas, after establishment of the 'steady state', this ratio had decreased to slightly over 2. This showed that at the earliest times succinate could not have been an isotopic precursor of malate, but that isotope from the added [2-^{14}C]acetate must have entered malate without passing through the stage of succinate. These results, which are similar to those reported for *Pseudomonas* KB1 (Kornberg, 1958) therefore offer presumptive evidence for the operation of the glyoxylate cycle in *A. niger* growing in acetate.

Incorporation of [1-^{14}C]*acetate by extracts of* Aspergillus niger. Cell-free extracts of acetate-grown *Aspergillus* catalysed the formation of

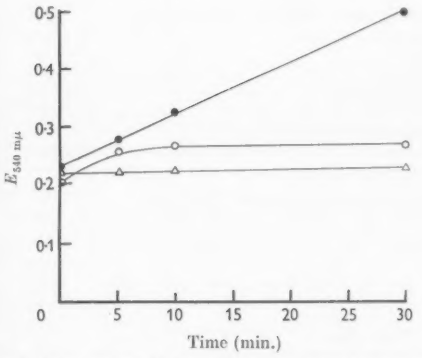

Fig. 3. Time course of the formation of acetohydroxamic acid by extracts of acetate-grown *Aspergillus niger*. For conditions, see Methods section. ●, Complete system; ○, acetate omitted; △, coenzyme A omitted.

acetyl-coenzyme A from acetate, ATP and coenzyme A (Fig. 3). The formation of acetyl-coenzyme A, measured as acetohydroxamic acid in the colorimetric assay of Jones & Lipmann (1955), depended on the presence of acetate and coenzyme A and proceeded linearly with time over the period of incubation (30 min.). The activity of acetic thiokinase in the extracts fell rapidly on storage: the most active preparation tested, assayed less than 1 hr. after harvesting of the cells, catalysed the formation of 1·74 μmoles of acetohydroxamic acid/mg. dry wt. of cells/hr., which (if the activity of this enzyme were the rate-limiting step in growth on acetate) would allow a doubling of cell mass in about 12 hr. and is of the order of the observed mean generation time (9·1 hr.).

When [1-^{14}C]acetate was added to cell extracts supplemented with ATP, magnesium chloride, glutathione and coenzyme A, small amounts (4–9 μm-moles) of radioactive citrate, malate, succinate and fumarate were formed (Table 1). Further supplementation of this mixture with α-oxoglutarate, succinate, fumarate or pyruvate did not promote any major increase in the amounts of isotope incorporated. However, addition of oxaloacetate resulted in the formation of 816 μm-moles of labelled citrate and 90 μm-moles of labelled glutamate, which indicated that the extract contained the citrate-forming condensing enzyme (Ochoa, Stern & Schneider, 1951) and probably also some aconitase, *iso*citric dehydrogenase and either glutamic dehydrogenase or α-oxoglutaric–amino acid transaminases. A similarly large incorporation of isotope from acetate was effected by addition of glyoxylate: the content of labelled malate rose from 9 to 822 μm-moles and that of fumarate from 5 to 187 μm-moles. This indicated the presence in the extract of fumarase and of one of the key enzymes of the glyoxylate cycle, malate synthetase (Wong & Ajl, 1956; Dixon, Kornberg & Lund, 1960) which catalyses the reaction (i) shown at foot of page.

A major incorporation of isotope into malate was also observed when *iso*citrate was added to the reaction mixture: 234 μm-moles of labelled malate and 50 μm-moles of labelled fumarate were formed, these compounds being the only labelled products of the reaction. This indicates that the extract contained, in addition to malate synthetase, the other enzyme unique to the glyoxylate cycle, *iso*citratase (Campbell, Smith & Eagles, 1953; Smith & Gunsalus, 1955, 1957; Olson, 1954, 1959; Saz, 1954; Saz & Hillary, 1956), which catalyses the reaction (ii) shown at foot of page.

The combined effect of these reactions, reaction (iii) shown at foot of page, would explain the observed patterns of isotope incorporation in the presence of *iso*citrate or glyoxylate.

Malate synthetase and iso*citratase in extracts of acetate-grown* Aspergillus. Further evidence for the presence of malate synthetase in extracts of acetate-grown *Aspergillus* was obtained with experiments in which either [2-^{14}C]acetate or [1-^{14}C]glyoxylate was used as precursor of labelled malate (Table 2): little or no labelled malate was formed from labelled acetate in the absence of added unlabelled glyoxylate, or from labelled glyoxylate in the absence of unlabelled acetate, and only a mixture of the two substrates resulted in the formation of [^{14}C]malate.

Evidence for the presence of *iso*citratase in such extracts was obtained in several ways. When extracts were incubated as previously described with *iso*citrate, magnesium chloride and either glutathione (Kornberg & Madsen, 1957, 1958) or sodium sulphide (Kornberg & Beevers, 1957*a*, *b*) keto acids (as determined by the assay procedure of Friedemann & Haugen, 1943) were rapidly produced. Chromatographic analysis (El Hawary & Thompson, 1953) of the 2:4-dinitrophenylhydrazones of such keto acids showed that the material predominantly formed was glyoxylate, although small quantities of α-oxoglutarate were also produced. No glyoxylate was formed in the absence of either *iso*citrate or cell extract, or in the complete

[^{14}C]Acetyl-coenzyme A + glyoxylate + water → [^{14}C]malate + coenzyme A (i)

*iso*Citrate ⇌ succinate + glyoxylate (ii)

[^{14}C]Acetyl-coenzyme A + *iso*citrate + water → succinate + [^{14}C]malate + coenzyme A (iii)

Table 1. *Incorporation of isotope from* [1-^{14}C]*acetate catalysed by extracts of acetate-grown* Aspergillus niger

The incubation mixture contained 100 μmoles of potassium phosphate, pH 7·4, 10 μmoles of glutathione, 5 μmoles of magnesium chloride, 0·08 μmole of coenzyme A, 1·9 μmoles of sodium [1-^{14}C]acetate (containing 10 μc of isotope), 10 μmoles of potassium acetate, 5 μmoles of ATP, 0·2 ml. of freshly prepared extract (containing approx. 1·1 mg. of soluble protein), 10 μmoles of additional substrates as indicated and water to 1 ml. All tubes were incubated at 30° for 30 min., after which time 3 ml. of absolute ethanol was added. The labelled products formed were analysed as described by Kornberg & Madsen (1958).

Additional substrate (10 μmoles)	Quantities of labelled products formed (μm-moles)				
	Citrate	Succinate	Malate	Fumarate	Glutamate
None	4	8	9	5	0
None: boiled extract	2	0	2	0	0
Oxaloacetate	816	12	19	6	90
Citrate	6	9	30	10	5
L$_s$(+)-*iso*Citrate	8	9	234	50	0
α-Oxoglutarate	9	12	12	21	7
Glyoxylate	6	6	822	187	0
Succinate	5	3	7	6	0
Fumarate	8	0	0	0	3
Pyruvate	10	2	3	25	3

Table 2. *Formation of labelled malate from* [2-^{14}C]*acetate or* [1-^{14}C]*glyoxylate in extracts of acetate-grown* Aspergillus niger

The incubation mixture contained 100 μmoles of potassium phosphate, pH 7·4, 5 μmoles of magnesium chloride, 0·08 μmole of coenzyme A, 10 μmoles of glutathione, 5 μmoles of ATP, 0·2 ml. of cell extract (containing approx. 1·1 mg. of soluble protein), other substrates as indicated and water to 1 ml. All tubes were incubated at 30° for 30 min., after which time the reactions were terminated by the addition of 3 ml. of absolute ethanol. The labelled products were analysed as described by Kornberg & Madsen (1958).

Tube no.	Isotopic material added	Additional substrate	Quantities of labelled products (μm-moles)				
			Citrate	Glutamate	Fumarate	Malate	Succinate
1	Sodium [2-^{14}C]acetate (10 μc, 2·2 μmoles)	None	65	31	1	0	6
2	As 1	Glyoxylate (10 μmoles)	72	36	32	154	81
3	Sodium [1-^{14}C]glyoxylate (4·5 μc, 7·5 μmoles)	None	6	2	1	6	2
4	As 3	Acetate (10 μmoles)	60	80	100	234	69

Table 3. *Quantities of succinate and glyoxylate formed from* iso*citrate by extracts of acetate-grown* Aspergillus niger

The amounts of succinate formed from *iso*citrate (Expt. A) were estimated manometrically with succinoxidase from washed pigeon-breast muscle. The conditions of the incubation were similar to those described by Kornberg & Beevers (1957*b*) but glutathione was used as the thiol compound. *iso*Citrate was estimated spectrophotometrically as the extinction change at 340 mμ consequent upon the reduction of triphosphopyridine nucleotide by *iso*citrate and the *iso*citric dehydrogenase of *Pseudomonas* KB1 (Kornberg & Madsen, 1957, 1958). The amounts of glyoxylate formed from *iso*citrate (Expt. B) were measured spectrophotometrically as the total change of extinction at 252 mμ, pH 6, consequent upon the formation of glyoxylic acid semicarbazone (Olson, 1959). The semicarbazone formed was decomposed with 6 N-HCl and 2:4-dinitrophenylhydrazine hydrochloride; chromatography (El Hawary & Thompson, 1953) of the 2:4-dinitrophenylhydrazones showed that of glyoxylic acid to be the only one formed.

Expt.	*A. niger* extract	*iso*Citrate (μmoles) present at		*iso*Citrate utilized (μmoles)	Succinate present at		Succinate formed (μmoles)	Glyoxylate formed (μmole)
		0 min.	30 min.		0 min.	30 min.		
A	Fresh	46·4	32·5	13·9	6·8	19·9	13·1	—
A	Boiled	45·9	45·7	0·2	6·8	6·8	0	—
B	Fresh	3·28	2·43	0·85	—	—	—	0·81
B	Boiled	3·28	3·28	0	—	—	—	0

system if the enzyme was boiled before incubation. Addition of purified aconitase (kindly donated by Dr W. Bartley) and [2-^{14}C]citrate (eluted from chromatograms after reaction of [2-^{14}C]acetate and oxaloacetate, as described in Table 1) further showed that the appearance of glyoxylate was also accompanied by the formation of [^{14}C]succinate as the only labelled product, as expected from the stoicheiometry of reaction (ii).

These results, although providing evidence for the presence of *iso*citratase, were not sufficient for a quantitative estimate of the activity of this enzyme. Unlike the behaviour of extracts of *Pseudomonas* (Smith & Gunsalus, 1957; Kornberg & Madsen, 1957, 1958) or castor beans (Kornberg & Beevers, 1957*a*, *b*), extracts of *Aspergillus* appeared to contain enzymes which destroyed glyoxylate rapidly and without concomitant gas uptake or evolution. The action of the enzyme system found in *A. niger* by Franke & de Boer (1958), which catalyses the dismutation of glyoxylate to oxalate and glycollate, would account for this destruction of glyoxylate. It was thus impossible to obtain from such extracts any quantitative relation between the rates and amounts of *iso*citrate disappearance and glyoxylate formation. Attempts to purify the *iso*citratase by the method described for *Pseudomonas* by Smith & Gunsalus (1957) were unsuccessful: although considerable purification was achieved, the enzyme was rapidly destroyed on storage.

Use was therefore made of the assay procedure of Olson (1959), of which Dr Olson kindly provided details before publication. This technique employs the trapping of glyoxylate formed from *iso*citrate at pH 6 with semicarbazide, the rate of formation of the –C=N– linkage of the semicarbazone being measured spectrophotometrically at 252 mμ. By these means, it was shown that at least 1·04 μmoles of glyoxylate/hr./mg. dry wt. of crushed mould was formed in fresh extracts of acetate-grown *Aspergillus*: since the activity of *iso*citratase is considerably lower at pH 6 than under more alkaline conditions (under which, however, the speed of condensation of glyoxylate and semicarbazide would impose a rate-limiting step), it is likely that the observed value is a minimum one but is sufficient to allow of a doubling of cell mass in 9 hr. When the semicarbazone formed in this reaction was decomposed by incubation with 2:4-dinitrophenylhydrazine in 6N-hydrochloric acid, and the resultant 2:4-dinitrophenylhydrazones were extracted and analysed by paper chromatography (El Hawary & Thompson, 1953), glyoxylate was found to be the only keto compound formed from *iso*citrate. Estimation of the amounts of succinate formed from *iso*citrate, and the amounts of glyoxylate produced under conditions under which the glyoxylate was trapped (Table 3) further confirmed that for each mol.prop. of *iso*citrate utilized, one mol.prop. of glyoxylate and one of succinate were produced.

Enzymes of the glyoxylate cycle in extracts of Aspergillus niger *grown on compounds other than acetate.* Cell-free extracts of *A. niger* which had been grown on the usual growth medium but in which the carbon source was glucose contained less than 5% of the acetic-thiokinase activity found in extracts of acetate-grown *A. niger*: although the latter extracts catalysed the formation of 1·7 μmoles of acetohydroxamic acid/hr./mg. dry wt. of cells, the former formed only 0·04 μmole of acetohydroxamic acid/hr./mg. dry wt. of cells. This low activity was also reflected in the decreased ability of extracts of glucose-grown cells to catalyse the incorporation of isotope from [1-^{14}C]acetate (Table 4). However, the small amounts of radioactive materials formed from acetate are adequate to show that both the citrate-forming condensing enzyme and malate synthetase were present: addition of oxaloacetate promoted the formation of 35 μm-moles of labelled citrate, and addition of glyoxylate that of 10 μm-moles of labelled malate. Addition of *iso*citrate did not stimulate the incorporation of isotope into malate, which indicated that the extract contained little or no *iso*citratase. This was confirmed by spectrophotometric assay (Olson, 1959): whereas extracts of the acetate-grown mould catalysed the formation of 1·04 μ-moles of glyoxylate/hr./mg. dry wt. of crushed mould, extracts of glucose-grown cells formed less than 0·007 μmole/hr./mg. dry wt. and those of succinate-grown cells less than 0·02. This indicates that in *A. niger*, as in other micro-organisms (Smith & Gunsalus, 1955, 1957; Olson, 1959; Callely, Dagley & Hodgson, 1958; Kornberg, Gotto & Lund, 1958; Kornberg & Lund, 1959; Kornberg *et al.* 1960*a*; Kornberg, Phizackerley & Sadler, 1960*b*) *iso*citratase is formed only under conditions

Table 4. *Incorporation of isotope from* [1-^{14}C]*acetate catalysed by extracts of glucose-grown* Aspergillus niger

The cell extract used was obtained from *A. niger* grown on the usual growth medium but in which ammonium acetate had been replaced by 50 mM-glucose, 50 mM-ammonium chloride and 0·05% of Difco yeast extract. Each tube received 0·2 ml. of extract (containing 1·0 mg. of soluble protein). Other conditions are as in Table 1.

Additional substrate (10 μmoles)	Labelled products formed (μm-moles)			
	Succinate	Malate	Citrate	Fumarate
None	11	12	0	9
Oxaloacetate	10	11	35	7
Glyoxylate	9	22	0	9
*iso*Citrate	13	10	0	7

necessitating the net formation of cellular materials from acetate, and thus supports the postulated role of the glyoxylate cycle in the growth of the organism on acetate. Malate synthetase was found to be present in extracts of *Aspergillus* under all conditions of growth.

DISCUSSION

The oxidation of acetate by *A. niger* is known to proceed via the tricarboxylic acid cycle (Martin, 1954; Ramakrishnan & Martin, 1954*a*, *b*; Ramakrishnan, 1954). This pathway provides both energy to the cell and intermediates, the carbon skeletons of which are the precursors of cell constituents (Krebs, Gurin & Eggleston, 1952; Roberts, Abelson, Cowie, Bolton & Britten, 1955; Ehrensvärd, 1955). Growth of the organism on acetate as sole carbon source therefore demands the occurrence of reactions effecting the net synthesis of intermediates of the tricarboxylic acid cycle from acetate.

One such route for the replenishment from acetate of the intermediates drained from the cycle during growth might be the direct oxidative condensation of acetate, to yield succinate, reaction (iv)

$$2 \text{ acetate} \rightarrow \text{succinate} + 2\text{H} \qquad \text{(iv)}$$

postulated by Thunberg (1920), Wieland (1922) and Knoop (1923). Although attempts to demonstrate this reaction with purified enzymes have been unsuccessful, it has been widely invoked to account for the growth of micro-organisms on C_2 compounds (for reviews, see Foster, 1949, 1958; Ajl, 1951, 1958; Chain, 1955; Kornberg, 1959). In particular, a reaction of this kind was required to explain the distribution of isotope within compounds synthesized by fungi growing on isotopically labelled acetate (Lewis & Weinhouse, 1951*a*, *b*) or ethanol (Foster & Carson, 1950; Foster *et al.* 1949).

A second route is the glyoxylate cycle (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957), the overall reaction of which is identical with reaction (iv) and the operation of which, in conjunction with the tricarboxylic acid cycle, would satisfactorily account for the isotope distribution patterns recorded (for review see Kornberg, 1959).

The evidence presented in this paper favours the second alternative. Incubation of *A. niger*, growing on [2-^{14}C]acetate for brief periods, showed that malate acquired isotope from acetate at the earliest times and without passing through the stage of succinate. The proportion of the total incorporated radioactivity present in malate fell with time, whereas that in succinate rose: this behaviour conforms to that expected from the operation of the glyoxylate cycle, and excludes a direct formation of succinate from acetate. Of the key enzymes of the glyoxylate cycle, *iso*citratase had already been shown to occur in the fungi *Rhizopus nigricans* and *Penicillium chrysogenum* by Olson (1954), who termed the enzyme '*iso*citric lyase'. Assay of the enzymic activity of extracts of *A. niger* confirm this finding: extracts of the acetate-grown mould contain *iso*citratase activity at pH 6 sufficient to allow of a doubling of cell mass in about 10 hr. were the activity of this enzyme to impose the rate-limiting step of growth on acetate. Since the activity of *iso*citratase at pH 6 is less than a tenth of that at pH 7 (Smith & Gunsalus, 1957), it is probable that the measured value is a minimum one, and that the activity of *iso*citratase is therefore more than adequate to account for the doubling of cell mass in 9·1 hr. observed during growth on acetate. The virtual absence of this enzyme in extracts of *A. niger* grown under conditions not necessitating the net synthesis of tricarboxylic acid cycle intermediates from acetate supports the view that *iso*citratase plays an essential role in the growth of the mould on acetate.

A second key enzyme of the glyoxylate cycle, malate synthetase, was discovered in extracts of *E. coli* by Wong & Ajl (1956). Its presence in extracts of *A. niger* is shown by the ability of such extracts, supplemented with cofactors, to catalyse the formation of malate from either [^{14}C]acetate and glyoxylate or [^{14}C]glyoxylate and acetate. The amounts of malate formed were similar to those of citrate produced from [^{14}C]acetate and oxaloacetate. Although no quantitative assay for malate synthetase was available at the time of completion of this work, these results indicate that the activities of the citrate-forming condensing enzyme and of malate synthetase were of the same order of magnitude. The presence of this enzyme (in the virtual absence of *iso*citratase) in extracts of *A. niger* grown on glucose or succinate further indicates that in this mould, as in *Pseudomonas* (Kornberg *et al.* 1958; Kornberg & Lund, 1959), *Micrococcus denitrificans* (Kornberg *et al.* 1960*a*) and *Saccharomyces drosophilarum* (Barnett & Kornberg, 1960), the operation of the glyoxylate cycle is controlled by factors influencing the intracellular activity and/or the formation of *iso*citratase.

SUMMARY

1. Isotope from [2-^{14}C]acetate added to *Aspergillus niger* growing on acetate as the sole carbon source was rapidly incorporated into cellular components. At the earliest times, only intermediates of the tricarboxylic acid cycle and amino acids derived therefrom became labelled.

2. Isotope from acetate entered the tricarboxylic acid cycle at two sites, to form citrate at one and

malate at the other. At the earliest times, labelled malate could not have been solely derived from labelled succinate. These results are consistent with the simultaneous operation of the tricarboxylic acid and glyoxylate cycles.

3. These findings also exclude the formation of succinate from the oxidative condensation of acetate as being of quantitative importance in the growth of *A. niger* on acetate.

4. Cell-free extracts of the acetate-grown mould contained the two key enzymes of the glyoxylate cycle, *iso*citratase and malate synthetase, in quantities adequate to account for the observed rates of growth of the organism. When supplemented with cofactors, such extracts also catalysed the overall formation of malate and succinate from acetate and *iso*citrate.

5. Cell-free extracts of the mould grown on glucose or succinate contained malate synthetase but only traces of *iso*citratase. This indicates that the operation of the glyoxylate cycle is controlled by factors influencing the intracellular activity of *iso*citratase.

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REFERENCES

Ajl, S. J. (1951). *Bact. Rev.* **15**, 211.
Ajl, S. J. (1958). *Physiol. Rev.* **38**, 196.
Aubert, J.-P., Milhaud, G. & Millet, J. (1956). *C.R. Acad. Sci., Paris*, **242**, 2059.
Aubert, J.-P., Milhaud, G. & Millet, J. (1957). *Ann. Inst. Pasteur*, **92**, 515.
Barnett, J. A. & Kornberg, H. L. (1960). *J. gen. Microbiol.* **23**, 65.
Callely, A. G., Dagley, S. & Hodgson, B. (1958). *Biochem. J.* **69**, 173.
Calvin, M. (1955). *Proc. 3rd int. Congr. Biochem., Brussels*, p. 211.
Campbell, J. J. R., Smith, R. A. & Eagles, B. A. (1953). *Biochim. biophys. Acta*, **11**, 594.
Chain, E. B. (1955). *Proc. 3rd int. Congr. Biochem., Brussels*, p. 523.
Dixon, G. H., Kornberg, H. L. & Lund, P. (1960). *Biochim. biophys. Acta*, **41**, 217.
Ehrensvärd, G. (1955). *Annu. Rev. Biochem.* **24**, 275.
El Hawary, M. F. S. & Thompson, R. H. S. (1953). *Biochem. J.* **53**, 340.
Foster, J. W. (1949). *Chemical Activities of Fungi*. New York: Academic Press Inc.
Foster, J. W. (1958). *Tex. Rep. Biol. Med.* **16**, 79.
Foster, J. W. & Carson, S. F. (1950). *Proc. nat. Acad. Sci., Wash.*, **36**, 219.
Foster, J. W., Carson, S. F., Anthony, D. S., Davis, J. B., Jefferson, W. E. & Long, M. V. (1949). *Proc. nat. Acad. Sci., Wash.*, **35**, 663.
Franke, W. & de Boer, W. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, no. 10–83, p. 134.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
Jones, M. E. & Lipmann, F. (1955). In *Methods in Enzymology*, vol. 1, p. 585. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Knoop, F. (1923). *Klin. Wschr.* **2**, 60.
Kornberg, A. (1955). In *Methods in Enzymology*, vol. 1, p. 705. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Kornberg, H. L. (1958). *Biochem. J.* **68**, 535.
Kornberg, H. L. (1959). *Annu. Rev. Microbiol.* **13**, 49.
Kornberg, H. L. & Beevers, H. (1957*a*). *Nature, Lond.*, **180**, 35.
Kornberg, H. L. & Beevers, H. (1957*b*). *Biochim. biophys. Acta*, **26**, 531.
Kornberg, H. L. & Collins, J. F. (1958). *Biochem. J.* **68**, 3P.
Kornberg, H. L., Collins, J. F. & Bigley, D. (1960*a*). *Biochim. biophys. Acta*, **39**, 13.
Kornberg, H. L., Gotto, A. M. & Lund, P. (1958). *Nature, Lond.*, **182**, 1430.
Kornberg, H. L. & Krebs, H. A. (1957). *Nature, Lond.*, **179**, 988.
Kornberg, H. L. & Lund, P. (1959). *Biochem. J.* **72**, 33P.
Kornberg, H. L. & Madsen, N. B. (1957). *Biochim. biophys. Acta*, **24**, 651.
Kornberg, H. L. & Madsen, N. B. (1958). *Biochem. J.* **68**, 549.
Kornberg, H. L., Phizackerley, P. J. R. & Sadler, J. R. (1960*b*). *Biochem. J.* **77**, 438.
Krebs, H. A. (1937). *Biochem. J.* **31**, 2095.
Krebs, H. A., Gurin, S. & Eggleston, L. V. (1952). *Biochem. J.* **51**, 614.
Lewis, K. F. & Weinhouse, S. (1951*a*). *J. Amer. chem. Soc.* **73**, 2500.
Lewis, K. F. & Weinhouse, S. (1951*b*). *J. Amer. chem. Soc.* **73**, 2906.
Martin, S. M. (1954). *Canad. J. Microbiol.* **1**, 6.
Ochoa, S., Stern, J. R. & Schneider, M. C. (1951). *J. biol. Chem.* **193**, 691.
Olson, J. A. (1954). *Nature, Lond.*, **194**, 695.
Olson, J. A. (1959). *J. biol. Chem.* **234**, 5.
Ramakrishnan, C. V. (1954). *Enzymologia*, **17**, 169.
Ramakrishnan, C. V. & Martin, S. M. (1954*a*). *Canad. J. Biochem. Physiol.* **32**, 434.
Ramakrishnan, C. V. & Martin, S. M. (1954*b*). *Arch. Biochem. Biophys.* **55**, 403.
Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). *Studies of Biosynthesis in* Escherichia coli. Washington: Carnegie Institution of Washington, Publ. no. 607.
Santer, M. & Vishniac, W. (1955). *Biochim. biophys. Acta*, **18**, 157.

Saz, H. J. (1954). *Biochem. J.* **58**, xx.
Saz, H. J. & Hillary, E. P. (1956). *Biochem. J.* **62**, 563.
Smith, R. A. & Gunsalus, I. C. (1955). *Nature, Lond.*, **175**, 774.
Smith, R. A. & Gunsalus, I. C. (1957). *J. biol. Chem.* **229**, 305.
Thunberg, T. (1920). *Skand. Arch. Physiol.* **40**, 1.
Trudinger, P. A. (1955). *Biochim. biophys. Acta*, **18**, 581.
Trudinger, P. A. (1956). *Biochem. J.* **64**, 274.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques and Tissue Metabolism*, 3rd ed. p. 202. Minneapolis: Burgess Publishing Co.
Wieland, H. (1922). *Ergebn. Physiol.* **20**, 477.
Wong, D. T. O. & Ajl, S. J. (1956). *J. Amer. chem. Soc.* **78**, 3230.

Biochem. J. (1960) **77**, 438

The Metabolism of C_2 Compounds in Micro-organisms

5. BIOSYNTHESIS OF CELL MATERIALS FROM ACETATE IN *ESCHERICHIA COLI**

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The tricarboxylic acid cycle (Krebs & Johnson, 1937) has been shown to be the major route for the oxidation of acetate by *Escherichia coli* (Swim & Krampitz, 1954; Gilvarg & Davis, 1956) and for the provision of the carbon skeletons of cell constituents (Roberts, Abelson, Cowie, Bolton & Britten, 1955; Gilvarg & Davis, 1956). When the organisms grow on acetate as sole carbon source and intermediates are removed from the cycle to supply cell materials, reactions must occur to replenish from acetate the supply of such intermediates. It has been postulated that this may be effected by condensation of acetate with carbon dioxide, to yield a C_3 compound which, by further carboxylation, yields malate or oxaloacetate (Abelson, Bolton, Britten, Cowie & Roberts, 1954; Roberts *et al.* 1955), or by the direct formation of succinate (Thunberg, 1920) from the oxidative condensation of 2 molecules of acetate (Glasky & Rafelson, 1957, 1959; Glasky, Eichholz & Rafelson, 1958). A third possible route is the glyoxylate cycle (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957).

The purpose of this paper is to show that isotope from [1-^{14}C]acetate is incorporated into cellular materials by *E. coli* strain *w*, growing on acetate, in a manner consistent with the simultaneous operation of the tricarboxylic acid and glyoxylate cycles, and that extracts of such cells contain the requisite enzymes in high activity. No evidence for the postulated successive carboxylation of acetate or for the direct formation of succinate was obtained. Since this latter finding might have been due to species differences, these experiments were repeated with *E. coli*, Crooks strain, as used by Glasky & Rafelson (1957, 1959) and Glasky *et al.* (1958). The results obtained again exclude the direct formation of succinate from acetate. They support the view that acetate is catabolized via the tricarboxylic acid cycle, and that the glyoxylate cycle operates as a means of supplying intermediates to that cycle. This work has been presented in part to The Biochemical Society (Kornberg, Phizackerley & Sadler, 1959).

* Part 4: Collins & Kornberg (1960).

METHODS

Maintenance and growth of organisms. A culture of *E. coli* strain *w* was the gift of Professor B. D. Davis (Harvard University Medical School, Boston, Mass., U.S.A.). *E. coli*, Crooks strain, was obtained from The National Collection of Industrial Bacteria, Teddington, Middx. (catalogue no. 8545).

The organisms were maintained on agar slopes consisting of 50 mM-potassium phosphate buffer, pH 7·2, 50 mM-ammonium chloride, 50 mM-sodium acetate, essential salts (4 mg. of $CaCl_2,6H_2O$; 8 mg. of $MgSO_4,7H_2O$; 0·4 mg. of $MnSO_4,4H_2O$; 0·4 mg. of $FeSO_4,7H_2O$/100 ml. of medium), solidified with 2% (w/v) of agar agar (Hopkins and Williams Ltd., Chadwell Heath, Essex). Stock cultures of the organism were subcultured every 2 weeks, grown at 30° and stored at 2°.

For growth in liquid medium, a loopful of organisms from a freshly grown slope was suspended in a Carrel culture flask (J. A. Jobling and Co. Ltd., Sunderland) containing 400 ml. of the above-mentioned medium but with the agar agar omitted. The flasks were shaken at 30° for 16–24 hr. on a reciprocal shaker. Growth of the cells was determined by measurements in an EEL nephelometer (Evans Electroselenium Ltd., Harlow, Essex) of the light-scattering of samples of the bacterial suspensions and comparison of the observed readings with a previously constructed calibration curve relating such readings to the dry

weight of bacteria/ml. of suspension. Although the medium was adequate to sustain growth of over 1 mg. dry wt./ml., the bacteria were removed while still in the phase of logarithmic growth, at densities of 0·2–0·4 mg. dry wt./ml. The contents of the Carrel flasks were centrifuged at 10° for 15 min. at 1500 *g*.

Bacterial incorporation of [1-^{14}C]*acetate.* (*a*) Experiments with *E. coli*, strain *w*. Bacteria that had been centrifuged after growth in Carrel culture flasks were suspended without washing in fresh 50 mM-acetate growth medium, and the vigorously aerated cell suspension was placed in a water bath at 30°. Growth was followed for about an hour (mean generation time, 130 min.).

In those experiments in which the cells were to be incubated with the isotope for 15 sec. or less, 1·0 ml. samples of a suspension of growing cells, at 3·1 mg. dry wt./ml., were drawn into a hypodermic syringe containing 0·4 ml. of [1-^{14}C]acetate (120 μc/ml.; 20 mM) and 0·6 ml. of air; they were shaken manually and then ejected after a measured time interval into tubes containing 3·0 ml. of absolute ethanol.

In those experiments in which the cells were incubated with isotope for 15 sec. or longer, the procedure of Kornberg (1958) was followed, except that the incubation was terminated by transfer of the samples of bacterial suspension to centrifuge tubes chilled in crushed ice. The samples were quickly centrifuged in the high-speed head of an International refrigerated centrifuge (25 000 *g*) at 0°, the supernatant solutions were discarded and the packed cells were immediately mixed with 3·0 ml. of absolute ethanol. Although this procedure introduces some uncertainty into the measurements of the times of sampling, and is thus unsuitable for very brief incubations, it has the advantage that most of the unassimilated isotope as well as the salts in the suspending medium are removed, and the resulting chromatograms are of better quality.

(*b*) Experiments with *E. coli*, Crooks strain. In order to reproduce as closely as possible the conditions used by Glasky & Rafelson (1959), but with shorter periods of incubation, the technique used was as described above, but the experiments were performed with thicker cell suspensions (about 8 mg. dry wt./ml.) at a lower incubation temperature (20°).

Analysis of samples obtained from incubation experiments. The treatment of the aqueous ethanolic suspension, the techniques of chromatographic analysis, of radioautography and radioassay, and of the methods of identification of labelled compounds, were as previously described (Kornberg, 1958).

Preparation of cell extracts. Extracts of cells were prepared in two ways: (*a*) by disintegration of washed packed cells in the Hughes (1951) press, and (*b*) by ultrasonic oscillation of suspensions of washed cells. Material obtained after crushing of cells was homogenized in 20 mM-potassium phosphate, pH 7·2, to which a single crystal of ribonuclease (L. Light and Co. Ltd., Colnbrook, Bucks) had been added, and the resulting suspension was centrifuged at 25 000 *g* at 0°. The supernatant solution, which contained 5–10 mg. of soluble protein/ml., was used for subsequent experiments.

Extracts of cells disrupted by ultrasonic oscillation were prepared by exposing suspensions of cells (10–30 mg. dry wt./ml.) in either 20 mM-potassium phosphate or 20 mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·2, to the output of a 600 W Mullard magnetostrictor oscillator, operating at 3·5 A, 25 kcyc./sec., for 2 min. The extracts were centrifuged at 25 000 *g* at 0° for 10 min. and the supernatant solutions were used.

Reactions of [1-^{14}C]*acetate in cell extracts.* Reactions of [1-^{14}C]acetate in extracts of *E. coli* were studied by the procedures previously described (Kornberg & Madsen, 1958; Collins & Kornberg, 1960).

Assays of L-*iso*citrate, *glyoxylate and malate.* L-*iso*Citrate was assayed by means of the triphosphopyridine nucleotide (TPN)-linked *iso*citric dehydrogenase obtained from extracts of glucose-grown *E. coli* (which contained negligible *iso*citratase). Glyoxylate was assayed as the phenylhydrazone, by measurement of light-absorption at 324 mμ (Dixon & Kornberg, 1959). Malate was assayed by the fluorescence method of Hummel (1949).

Assays of enzymes. Condensing enzyme (Ochoa, Stern & Schneider, 1951), malate synthetase (Wong & Ajl, 1956) and *iso*citratase (Smith & Gunsalus, 1954, 1957; Olson, 1954, 1959) were assayed by the spectrophotometric procedure of Dixon & Kornberg (1959).

Partial purification of isocitratase *and malate synthetase.* In order better to demonstrate the net formation of malate from acetyl-coenzyme A and *iso*citrate, extracts of *E. coli*, strain *w*, were partially purified to remove interfering enzymes (e.g. aconitase, *iso*citric dehydrogenase, phosphotransacetylase). Packed cells (35 g. wet wt.) were suspended in 3 vol. of 50 mM-potassium phosphate, pH 7·2, and disrupted by sonic oscillation. The resulting 120 ml. of solution was centrifuged at 25 000 *g* in an International refrigerated centrifuge at 0° for 15 min. The supernatant solution was treated with 10 ml. of 2% (w/v) protamine sulphate to remove nucleic acids, and was fractionated with ammonium sulphate. The fractions precipitated between 40 and 55% saturation (rich in *iso*citratase), and between 55 and 70% saturation (rich in malate synthetase), were dissolved in 20 ml. of 20 mM-potassium phosphate, pH 7·2, and dialysed overnight at 3° in 10 l. of the same buffer containing in addition 0·1 g. of cysteine–HCl. The clear solutions after dialysis were used experimentally.

Materials. Potassium dihydrogen L$_s$-*iso*citrate and highly purified sodium glyoxylate were gifts from Dr H. B. Vickery and Dr I. Zelitch respectively (Connecticut Agricultural Experimental Station, New Haven, Conn., U.S.A.). Pyridine nucleotides and coenzyme A were purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A. Other chemicals used were of A.R. grade. Isotopic materials were obtained from The Radiochemical Centre, Amersham, Bucks.; they were purified before use as described by Kornberg (1958).

RESULTS

Utilization of [1-^{14}C]*acetate.* When [1-^{14}C]acetate was added to suspensions of *E. coli* growing on acetate, isotope was rapidly incorporated into cellular materials soluble in aqueous ethanol. The rates of incorporation of ^{14}C were constant over the first minute and corresponded to the uptake respectively of 12·6 μmoles of acetate/mg. dry wt. of cells/hr. for strain *w* at 30° (Fig. 1, *A*) and 7·6 μmoles/mg. dry wt. of cells/hr. for the Crooks strain at 20° (Fig. 1, *B*). These values are minimum ones,

since neither volatile products (such as carbon dioxide) nor chromatographically fugitive materials (such as oxaloacetate or pyruvate) were assayed, but the rates of entry of acetate are of the order required (9 μmoles/hr./mg. dry wt. of cells) to account for the observed rates of growth of *E. coli* on acetate at 30° (mean generation time: 130 min.).

Distribution of ^{14}C *amongst the labelled constituents of the soluble fraction.* Two-dimensional chromatography and radioautography of the fractions soluble in aqueous ethanol, obtained from each sample of the growing suspensions, showed that ^{14}C from acetate rapidly appeared in many intermediates of the tricarboxylic acid cycle and in amino acids directly derived therefrom. Even at the shortest times of incubation (3–5 sec.), isotope was detected in citrate, glutamate, malate and aspartate, with traces also in succinate and fumarate. These last-named compounds were only partially separated by the chromatographic procedures used. Phosphorylated compounds became appreciably radioactive only after longer times of incubation (more than 1 min.), which indicated that carbon dioxide-fixing reactions, such as operate in photosynthetic (Calvin, 1955) or autotrophic (Santer & Vishniac, 1955; Trudinger, 1955, 1956) organisms, were not of quantitative importance in the growth of *E. coli* on acetate.

When the proportion of the total radioactivity contributed by each labelled compound, expressed as the percentage of the total ^{14}C in the sample, was

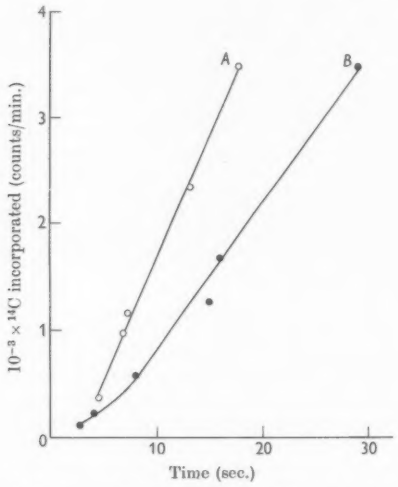

Fig. 1. Incorporation of ^{14}C from [1-^{14}C]acetate into the fractions soluble in aqueous ethanol of *E. coli* strain *w* (○) and Crooks (●) growing on acetate. For conditions, see Methods section.

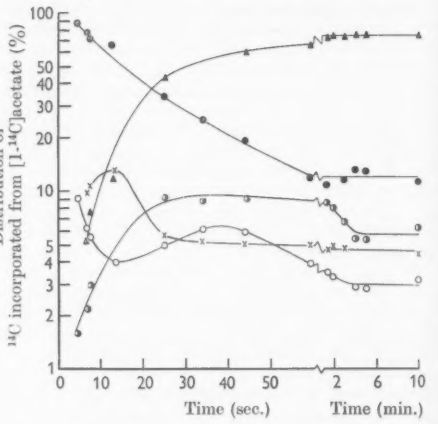

Fig. 2. Variation with time of the percentage distribution of ^{14}C incorporated from [1-^{14}C]acetate into the constituents of fractions soluble in aqueous ethanol of *E. coli*, strain *w*, growing on acetate. ●, Citrate; ○, malate; ▲, glutamate; ×, aspartate; ◑, (succinate + fumarate).

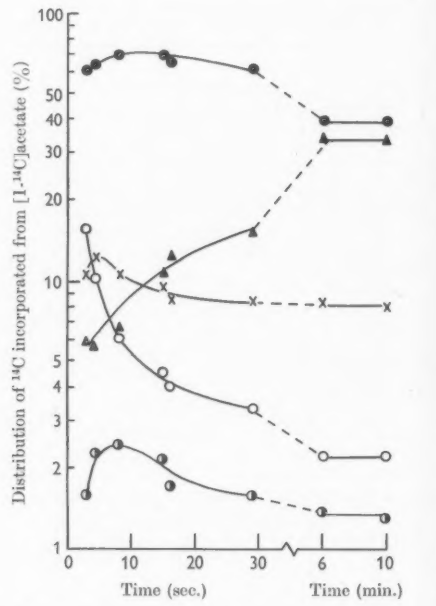

Fig. 3. Variation with time of the percentage distribution of ^{14}C incorporated from [1-^{14}C]acetate into the constituents of fractions soluble in aqueous ethanol of *E. coli*, Crooks strain, growing on acetate. ●, Citrate; ○, malate; ▲, glutamate; ×, aspartate; ◑, (succinate + fumarate).

plotted against time of incubation, smooth curves could be drawn through the points obtained (Figs. 2, 3). The general pattern of distribution of isotope amongst the labelled materials was similar for the two strains of organism tested. After an incubation of approx. 1 min., a 'steady state' of isotopic distribution had been reached, in which the proportion contributed by each labelled compound to the total radioactivity of the samples changed little with time. At and after this time, malate contained 3 % and 2·2 % respectively of the total ^{14}C incorporated into *E. coli*, strain *w*, and the Crooks strain. (Succinate plus fumarate) contained respectively 6 % and 1·5 % and citrate contributed 12 % and 39 % to the total radioactivity. The ratio of the isotope present in the malate to that present in (succinate plus fumarate) also remained constant after the 'steady state' had been reached: ratios of 1:2 were observed with strain *w* and 2:1 with the Crooks strain (Fig. 4).

At the earliest times of incubation, these isotope-distribution patterns were markedly different. Whereas the proportion of label contributed by (succinate + fumarate) rose with time, that of malate fell rapidly in both strains of *E. coli* (Figs. 2, 3). Whereas malate contained approximately half the radioactivity of (succinate + fumarate) after the achievement of the 'steady state' in strain *w*, at the earliest times malate contained approximately six times as much label as did (succinate + fumarate) (Figs. 2 and 4). Similarly, the earliest samples taken from the Crooks strain contained over nine times as much labelled malate as they did labelled (succinate + fumarate) (Figs. 3, 4). In both strains, the greatest proportion of isotope incorporated at the earliest times was found in citrate, as expected from the operation of the tricarboxylic acid cycle.

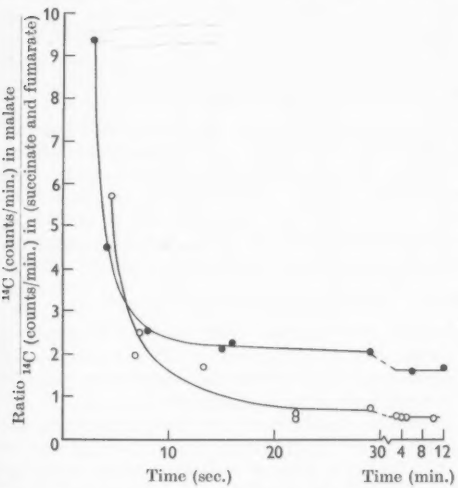

Fig. 4. Variation with time of the ratio of labelled malate to labelled (succinate + fumarate) formed from [1-^{14}C]-acetate by *E. coli* strains *w* (○) and Crooks (●) growing on acetate.

These results show that the direct formation of succinate from acetate, postulated by Glasky & Rafelson (1957, 1959) and Glasky *et al.* (1958), plays no quantitatively important role in the growth on acetate of either *E. coli* strain tested: the initially low and rising radioactivity of succinate precludes its primary formation from labelled acetate. The high proportion of isotope present in malate, and its rapid fall with time, shows also that succinate could not have been the major isotopic precursor of labelled malate but that labelled malate must have been formed from labelled acetate without passing through the stage of succinate.

It was conceivable that the high initial labelling of malate was due to fixation of labelled carbon dioxide (produced from the oxidation of [1-^{14}C]-acetate) by a C_3 compound also derived from acetate (Abelson *et al.* 1954; Roberts *et al.* 1955). This was tested by allowing 10 ml. of a suspension of *E. coli* strain *w* to grow on acetate in the presence of 1 ml. of 40 mM-sodium [^{14}C]bicarbonate (containing 200 μc of ^{14}C). Analysis of the samples as for the similar experiments with [1-^{14}C]acetate showed that the rate of incorporation of isotope was less than 2 % of that observed with a similar suspension of cells growing on [1-^{14}C]acetate with the same mean generation time; moreover, only a small proportion of the isotope incorporated from sodium [^{14}C]bicarbonate appeared in malate. These findings render it unlikely that *E. coli* growing on acetate derives cellular materials from successive carboxylations of acetate to C_3 and C_4 compounds or from the direct formation of succinate from acetate, but offer presumptive evidence for the operation of the glyoxylate cycle in the two strains tested.

Reactions of [1-^{14}C]*acetate in* Escherichia coli *extracts.* Evidence in support of the operation of the glyoxylate cycle was obtained when extracts of acetate-grown *E. coli* strain *w* [prepared in the Hughes (1951) press] were incubated with sodium [1-^{14}C]acetate, adenosine triphosphate, magnesium chloride, glutathione, coenzyme A and added unlabelled substrates. In the absence of such unlabelled substrates, only traces of non-volatile radioactive materials were formed (Table 1). Addition of oxaloacetate resulted in the formation of labelled citrate and a small quantity of labelled succinate. Addition of glyoxylate resulted in the formation of labelled malate and fumarate, which

Table 1. *Incorporation of isotope from* [1-^{14}C]*acetate into extracts of* Escherichia coli *strain* w

Incubation mixtures contained: 120 μmoles of potassium phosphate, pH 7·2; 10 μmoles of $MgCl_2$; 10 μmoles of adenosine triphosphate; 0·08 μmole of coenzyme A; 10 μmoles of glutathione; 4·5 μmoles of sodium [1-^{14}C]-acetate (containing 10 μc of ^{14}C and giving $6·5 \times 10^5$ counts/min. under the conditions of radioassay used); 0·10 ml. of an extract of crushed acetate-grown *E. coli* strain *w* (containing 0·9 mg. dry wt. of crushed cells); additional substrates as indicated and water to 1·0 ml. The mixtures were incubated for 30 min. at 30°. The reaction was terminated by addition of 3 ml. of ethanol and the labelled materials were analysed as previously described (Kornberg, 1958).

Additional substrates	$10^{-3} \times$ Radioactivity (counts/min.)			
	Citrate	Malate	Succinate	Fumarate
None	0·98	0·25	0·00	0·18
Oxaloacetate (10 μmoles)	13·4	0·18	0·76	0·35
Glyoxylate (10 μmoles)	0·51	12·0	0·20	3·13
*iso*Citrate (10 μmoles)	2·5	15·3	0·43	1·36
Diphosphopyridine nucleotide (0·4 mg.)	0·34	0·59	0·18	0·04

was also achieved when *iso*citrate was used instead of glyoxylate. These findings can be explained by the action of the key enzymes of the glyoxylate cycle. The citrate-forming condensing enzyme (Ochoa *et al.* 1951) catalyses the reaction (1)

$$[1\text{-}^{14}C]\text{Acetyl-coenzyme A} + \text{oxaloacetate} + \text{water} \rightarrow [1\text{-}^{14}C]\text{citrate} + \text{coenzyme A} \quad (1)$$

If the extract used also contained aconitase, part of the labelled citrate would isomerize to *iso*-citrate; subsequent cleavage of this material by *iso*citratase (2) (Smith & Gunsalus, 1954, 1957; Saz & Hillary, 1956; Olson, 1954, 1959) would explain the formation of the small amount of labelled succinate observed:

$$\textit{iso}\text{Citrate} \rightleftharpoons \text{succinate} + \text{glyoxylate} \quad (2)$$

The formation of labelled malate in the presence of unlabelled glyoxylate is due to the action of malate synthetase (3), discovered in extracts of *E. coli* by Wong & Ajl (1956):

$$[1\text{-}^{14}C]\text{Acetyl-coenzyme A} + \text{glyoxylate} + \text{water} \rightarrow [^{14}C]\text{malate} + \text{coenzyme A} \quad (3)$$

Enzymic removal of water, catalysed by fumarase, from the [^{14}C]malate thus formed would account for the observed formation of [^{14}C]fumarate from [^{14}C]acetate. The combined action of *iso*citratase (2) and malate synthetase (3) accounts for the observed formation of labelled malate when *iso*-citrate was added as unlabelled substrate (4).

$$[1\text{-}^{14}C]\text{Acetyl-coenzyme A} + \textit{iso}\text{citrate} + \text{water} \rightarrow [^{14}C]\text{malate} + \text{succinate} + \text{coenzyme A} \quad (4)$$

The addition of diphosphopyridine nucleotide did not stimulate the incorporation of isotope from [1-^{14}C]acetate: hence the extract did not catalyse the oxidative condensation of acetate to succinate (Seaman & Naschke, 1955).

Enzymes of the glyoxylate cycle in extracts of Escherichia coli. The presence of the enzymes which catalyse reactions (1)–(3) in extracts of both *E. coli* strain *w* and the Crooks strain was demonstrated with the spectrophotometric assays of Dixon & Kornberg (1959). In these, *iso*citratase activity was measured as the rate of increase of extinction at 324 mμ concomitant with the formation of glyoxylate phenylhydrazone from phenylhydrazine and glyoxylate produced by the aldol cleavage of *iso*citrate; the activities of malate synthetase and condensing enzyme were determined as the rate of decrease of extinction at 232 mμ consequent upon the cleavage of the acyl thio-ester bond of acetyl-coenzyme A in the presence of either glyoxylate or oxaloacetate. It was found (Table 2) that extracts of both strains of acetate-grown *E. coli* were rich in the three enzymes assayed. The specific activities (μmoles of substrate transformed/hr./mg. of soluble protein) of condensing enzyme averaged 23 for strain *w* and 14 for the Crooks strain; of malate synthetase 7 and 5·5 respectively; and of *iso*citratase 5·3 and 6·3 respectively. The activities of these enzymes, acting in conjunction with the other enzymes of the tricarboxylic acid cycle, are therefore sufficient to catalyse the net formation from acetate of at least 6 μmoles of C_4 compounds/hr./mg. of soluble protein. This value is a minimum one, as neither *iso*-citratase nor malate synthetase is at its optimum temperature or pH under the conditions of assay (Smith & Gunsalus, 1957; Dixon, Kornberg & Lund, 1960), but the value is of the order required to account for the observed rates of formation of cell material during growth on acetate.

Net formation of malate from acetate by extracts of acetate-grown Escherichia coli. That extracts of acetate-grown *E. coli* can catalyse the net formation of malate from acetyl-coenzyme A and *iso*citrate, via reaction (4), was demonstrated with partially purified *iso*citratase and malate synthetase obtained from *E. coli* strain *w* (Table 3). The quanti-

Table 2. *Average specific activities of key enzymes of the glyoxylate cycle in extracts of* Escherichia coli *strains* w *and Crooks*

Extracts were prepared by disintegration of washed cells, grown on the substrates indicated, for 2 min. in a 600 w Mullard magnetostrictor oscillator operating at 3·5 A, 25 kcyc./sec. The suspensions obtained were centrifuged at 0°, 25 000 *g* for 10 min.; enzymic assays were performed on the clear supernatant solutions by the procedures of Dixon & Kornberg (1959). Specific activities are expressed as μmoles of substrate transformed/mg. of soluble protein/hr.

Carbon source for growth	*E. coli* strain used	Average specific activity		
		*iso*Citratase	Malate synthetase	Condensing enzyme
Acetate	*w*	5·3	7·0	23
Acetate	Crooks	6·3	5·5	14
Succinate	*w*	0·47	0·55	1·4
Succinate	Crooks	0·70	—	—
Malate	*w*	0·64	0·39	1·3
Lactate	*w*	0·22	0·40	7·0

Table 3. *Formation of malate from acetyl-coenzyme A and* isocitrate

The complete system contained, in a silica cell (vol. 0·45 ml.), 70 μmoles of tris buffer, pH 7·2; 1 μmole of $MgCl_2$; 0·05 μmole of acetyl-coenzyme A (purified by electrophoresis and paper chromatography: Dixon, Kornberg & Lund, 1960); 0·02 μmole of cysteine–HCl; 1 μmole of L(+)-*iso*citrate; 0·02 ml. of partially purified *E. coli* extract (containing 20 units of *iso*citratase and 1 unit of malate synthetase) and water to 0·41 ml. A unit of enzyme is that quantity catalysing the transformation of 1 μmole of substrate/hr. under the conditions of assay described by Dixon & Kornberg (1959). The amounts of acetyl-coenzyme A utilized were calculated from the observed decrease in extinction at 232 mμ (Stadtman, 1957); the amounts of malate formed were determined by the fluorimetric procedure of Hummel (1949).

Expt. no.	Conditions	Time of incubation (min.)	Acetyl-coenzyme A utilized (μm-moles)	Malate formed (μm-moles)
1	*iso*Citrate omitted	10	0	0
1	Complete system	10	22	22
2	*iso*Citrate omitted	24	0	0
2	Enzyme omitted	24	0	0
2	Acetyl-coenzyme A omitted	24	—	0
2	Complete system	24	38	37

ties of acetyl-coenzyme A utilized by the extract incubated with excess of *iso*citrate were calculated from the measured decrease in extinction at 232 mμ (Stadtman, 1957); the amounts of malate formed were determined fluorimetrically (Hummel, 1949). It was found that for each μmole of acetyl-coenzyme A split an equivalent amount of malate was formed.

Influence of growth substrates on enzymes of the glyoxylate cycle. Extracts prepared by ultrasonic disintegration of both strains of *E. coli* grown on carbon sources other than acetate contained only low activities of malate synthetase and *iso*citratase (Table 2), the specific activities of these enzymes being less than 10 % of those observed in extracts of the organisms which had been grown on acetate. The specific activity of condensing enzyme was also markedly decreased in proportion. These results, which differ from those reported for *Pseudomonas* (Kornberg, Gotto & Lund, 1958; Kornberg & Lund, 1959), *Micrococcus denitrificans* (Kornberg, Collins & Bigley, 1960) and *Aspergillus niger* (Collins & Kornberg, 1960), indicate that all the key enzymes of the glyoxylate cycle are under adaptive control in *E. coli* (cf. Wong & Ajl, 1957). The high activities of these enzymes in extracts of *E. coli* grown on acetate therefore provide further evidence for their involvement in the growth of the organisms on acetate.

DISCUSSION

The growth of micro-organisms on acetate as sole carbon source necessitates the occurrence of reactions, ancillary to the tricarboxylic acid cycle, which effect the net formation of intermediates of that cycle from acetate. Three possible routes whereby this might be achieved have been postulated for *E. coli*: (*a*) a direct formation of succinate from the oxidative condensation of 2 molecules of acetate; (*b*) successive carboxylations of acetate, to yield first a C_3 and then a C_4 compound; (*c*) the glyoxylate cycle.

'*Thunberg condensation*'. The formation of succinate by direct oxidative condensation of acetate was first postulated by Thunberg (1920), and is often referred to as the 'Thunberg condensa-

tion'. Although this reaction has been assumed to play a role in the metabolism of many micro-organisms (for review, see Ajl, 1951, 1958; Foster, 1949, 1958; Kornberg, 1959), it has not been possible to demonstrate convincingly its occurrence in cell-free extracts. There are several lines of evidence against its occurrence in *E. coli*.

Gilvarg & Davis (1956) isolated a mutant (M22–64) from *E. coli* strain *w* which lacked the citrate-forming condensing enzyme. This mutant was unable to oxidize acetate, although it readily oxidized succinate: no direct formation of succinate from acetate could therefore occur in this organism.

Swim & Krampitz (1954) incubated suspensions of *E. coli* anaerobically in the presence of fumarate and [2-^{13}C]acetate. Under these conditions succinate accumulates by the overall reaction (5):

$$\text{Acetate} + 4\ \text{fumarate} + 2\ \text{water} \rightarrow 4\ \text{succinate} + 2\ \text{carbon dioxide}, \quad (5)$$

as first established by Krebs (1937). The evolved carbon dioxide was not enriched with ^{13}C, and ethylene, obtained from the decarboxylation of the succinate formed, was found to be a mixture of molecular species with masses 28 and 29. Had any succinate been formed via the 'Thunberg condensation' this succinate would have been labelled in both methylene groups and on chemical decarboxylation would have produced ethylene of configuration $^{13}CH_2{:}^{13}CH_2$ and mass 30. No such ethylene was detected, and hence the succinate arose from acetate via the reactions of the tricarboxylic acid cycle and not via the 'Thunberg condensation'. A similar explanation applies to the earlier work of Kalnitsky, Wood & Werkman (1943).

The results presented in the present paper confirm these findings. Both *E. coli* strain *w* and the Crooks strain incorporated isotope from [1-^{14}C]-acetate initially into citrate and malate. The primary formation of labelled citrate is expected from the operation of the tricarboxylic acid cycle. The heavy labelling of malate at times when succinate was only slightly radioactive (Fig. 4) indicates that succinate was not an isotopic precursor of malate at these times, and also excludes the 'Thunberg condensation' as being of quantitative importance in these strains of *E. coli*. It is possible that the results obtained by Glasky & Rafelson (1959), which are also not consistent with the operation of the tricarboxylic acid cycle, were vitiated by technical difficulties.

Carboxylation of acetate. The formation by *E. coli* of pyruvate or phosphopyruvate by carboxylation of acetate was postulated by Abelson *et al.* (1954) and Roberts *et al.* (1955). Although it is known that extracts of *E. coli* (Strecker & Ochoa, 1954) can catalyse the exchange of the carboxyl group of pyruvate with carbon dioxide, no evidence exists that such extracts catalyse the net formation of pyruvate from acetate and carbon dioxide. The results obtained by Bagatell, Wright & Sable (1958), in which isotope from [1-^{14}C]acetate was found to be incorporated significantly only into carbon atoms 3 and 4 of glucose synthesized by *E. coli* from acetate, further exclude such a carboxylation as being of quantitative importance. This is confirmed by the present work: isotope from added sodium [^{14}C]bicarbonate was incorporated into *E. coli* growing on acetate at less than 2% of the rate at which isotope from [1-^{14}C]acetate was incorporated.

Glyoxylate cycle. The overall reaction of the glyoxylate cycle (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957) is identical with that of the 'Thunberg condensation', but the mechanism is entirely different: acetate enters this cycle at two sites, to form citrate at one and malate at the other. The results presented in this paper are consistent with the operation of this scheme in both strains of *E. coli* studied. In particular, isotope from acetate was incorporated initially into citrate and malate, and the ratio of labelled malate to labelled succinate formed at early times precluded the formation of malate from labelled citrate via succinate. Cell-free extracts of the acetate-grown bacteria, supplemented with necessary cofactors, readily catalysed the formation of labelled citrate from labelled acetate and oxaloacetate, and of labelled malate from labelled acetate and either glyoxylate or *iso*citrate. Moreover, the cell-free extract catalysed the net formation of malate from acetyl-coenzyme A and *iso*citrate. Direct enzymic assay showed that the key enzymes of the glyoxylate cycle were present in such extracts in amounts adequate to account for the observed rates of growth of the organisms on acetate, and that growth on substrates other than acetate was accompanied by a marked decrease in the quantities of these enzymes formed. These findings are consistent with the view that the biosynthesis of cell materials from acetate by *E. coli* involves the necessary operation of both the tricarboxylic acid and glyoxylate cycles.

SUMMARY

1. In brief incubation periods, suspensions of two strains of *Escherichia coli* (*w* and Crooks) growing on acetate as the sole carbon source incorporated isotope from [1-^{14}C]acetate only into intermediates of the tricarboxylic acid cycle and into amino acids derived therefrom.

2. The distribution of isotope amongst the labelled products was not consistent with the

operation of the tricarboxylic acid cycle as the sole route of acetate metabolism, but showed that acetate entered the cycle at two sites, to form citrate at one and malate at the other. Succinate was not labelled at the earliest times. These results indicate that the glyoxylate cycle also operated in these cells, and exclude the primary formation of succinate by oxidative condensation of acetate.

3. Isotope from sodium [^{14}C]bicarbonate was incorporated by *E. coli* growing on acetate into cellular materials at less than 2% of the rate at which [1-^{14}C]acetate was utilized.

4. Cell-free extracts of acetate-grown cells, fortified with necessary cofactors, catalysed the incorporation of isotope from [1-^{14}C]acetate into citrate in the presence of oxaloacetate, and into malate in the presence of either glyoxylate or *iso*-citrate. They also catalysed the net formation of malate from acetyl-coenzyme A and *iso*citrate.

5. Direct enzymic assay showed that such extracts contained high activities of key enzymes of the glyoxylate cycle. The specific activities (μmoles of substrate transformed/mg. of soluble protein/hr.) of condensing enzyme averaged 23 for strain *w* and 14 for the Crooks strain, of malate synthetase 7·0 and 5·5, and of *iso*citratase 5·3 and 6·3 respectively. The activities of these enzymes, acting in conjunction with other enzymes of the tricarboxylic acid cycle, are adequate to account for the observed rates of growth of the organisms on acetate.

6. Cell-free extracts of *E. coli* grown on carbon sources other than acetate contained malate synthetase and *iso*citratase at less than one-tenth of the specific activities observed with acetate-grown cells. This supports the essential role of the glyoxylate cycle in the growth of *E. coli* on acetate.

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REFERENCES

Abelson, P. H., Bolton, E. T., Britten, R. J., Cowie, D. B. & Roberts, R. B. (1954). *Yearb. Carnegie Instn*, **53**, 77.
Ajl, S. J. (1951). *Bact. Rev.* **15**, 211.
Ajl, S. J. (1958). *Physiol. Rev.* **38**, 196.
Bagatell, F. K., Wright, E. W. & Sable, H. Z. (1958). *Biochim. biophys. Acta*, **28**, 216.
Calvin, M. (1955). *Proc. 3rd int. Congr. Biochem., Brussels*, p. 211.
Collins, J. F. & Kornberg, H. L. (1960). *Biochem. J.* **77**, 430.
Dixon, G. H. & Kornberg, H. L. (1959). *Biochem. J.* **72**, 3P.
Dixon, G. H., Kornberg, H. L. & Lund, P. (1960). *Biochim. biophys. Acta*, **41**, 217.
Foster, J. W. (1949). *Chemical Activities of Fungi*. New York: Academic Press Inc.
Foster, J. W. (1958). *Tex. Rep. Biol. Med.* **16**, 79.
Gilvarg, C. & Davis, B. D. (1956). *J. biol. Chem.* **222**, 307.
Glasky, A. J., Eichholz, A. & Rafelson, M. E. (1958). *Fed. Proc.* **17**, 230.
Glasky, A. J. & Rafelson, M. E. (1957). *Fed. Proc.* **16**, 186.
Glasky, A. J. & Rafelson, M. E. (1959). *J. biol. Chem.* **234**, 2118.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
Hummel, J. P. (1949). *J. biol. Chem.* **180**, 1225.
Kalnitsky, G., Wood, H. G. & Werkman, C. H. (1943). *Arch. Biochem.* **2**, 269.
Kornberg, H. L. (1958). *Biochem. J.* **68**, 535.
Kornberg, H. L. (1959). *Annu. Rev. Microbiol.* **13**, 49.
Kornberg, H. L., Collins, J. F. & Bigley, D. (1960). *Biochim. biophys. Acta*, **39**, 9.
Kornberg, H. L., Gotto, A. M. & Lund, P. (1958). *Nature, Lond.*, **182**, 1430.
Kornberg, H. L. & Krebs, H. A. (1957). *Nature, Lond.*, **179**, 988.
Kornberg, H. L. & Lund, P. (1959). *Biochem. J.* **72**, 33P.
Kornberg, H. L. & Madsen, N. B. (1957). *Biochim. biophys. Acta*, **24**, 651.
Kornberg, H. L. & Madsen, N. B. (1958). *Biochem. J.* **68**, 549.
Kornberg, H. L., Phizackerley, P. J. R. & Sadler, J. R. (1959). *Biochem. J.* **72**, 32P.
Krebs, H. A. (1937). *Biochem. J.* **31**, 2095.
Krebs, H. A. & Johnson, W. A. (1937). *Enzymologia*, **4**, 148.
Ochoa, S., Stern, J. R. & Schneider, M. C. (1951). *J. biol. Chem.* **193**, 691.
Olson, J. A. (1954). *Nature, Lond.*, **174**, 695.
Olson, J. A. (1959). *J. biol. Chem.* **234**, 5.
Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). *Studies of Biosynthesis in* Escherichia coli. Washington: Carnegie Institution of Washington, Publ. No. 607.
Santer, M. & Vishniac, W. (1955). *Biochim. biophys. Acta*, **18**, 157.
Saz, H. J. & Hillary, E. P. (1956). *Biochem. J.* **62**, 563.
Seaman, G. R. & Naschke, M. D. (1955). *J. biol. Chem.* **217**, 1.
Smith, R. A. & Gunsalus, I. C. (1954). *Nature, Lond.*, **175**, 774.
Smith, R. A. & Gunsalus, I. C. (1957). *J. biol. Chem.* **229**, 305.
Stadtman, E. R. (1957). In *Methods in Enzymology*, vol. 3, p. 931. New York: Academic Press Inc.
Strecker, H. J. & Ochoa, S. (1954). *J. biol. Chem.* **209**, 313.
Swim, H. E. & Krampitz, L. O. (1954). *J. Bact.* **67**, 426.
Thunberg, T. (1920). *Skand. Arch. Physiol.* **40**, 1.
Trudinger, P. A. (1955). *Biochim. biophys. Acta*, **18**, 581.
Trudinger, P. A. (1956). *Biochem. J.* **64**, 274.
Wong, D. T. O. & Ajl, S. J. (1956). *J. Amer. chem. Soc.* **78**, 3230.
Wong, D. T. O. & Ajl, S. J. (1957). *Science*, **126**, 1013.

Biochem. J. (1960) **77**, 446

The Determination of Cholesterol and Coprosterol in Faecal Lipids

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Snog-Kjaer, Prange & Dam (1956) and Coleman & Baumann (1957) have demonstrated the microbial conversion of cholesterol into coprosterol in the mammalian large intestine. Thus there is need for the rapid and accurate simultaneous quantitative determination of cholesterol and coprosterol. This is attempted in the chromatographic procedure of Coleman, Wells & Baumann (1956), which is too time-consuming for routine determinations.

The present paper describes a procedure for such routine determinations of cholesterol and coprosterol in faeces, based on a method originally published by Brown, Zlatkis, Zak & Boyle (1954) and more recently modified by Zak, Luz & Fisher (1957), for the determination of serum cholesterol. Both the original method and that described below employ a colour reagent consisting of ferric chloride–acetic acid–concentrated sulphuric acid, which produces a coloured cholesterol derivative with an absorption peak at 560 mμ. In the modification here described the absorption is measured at 440 as well as at 560 mμ, since coprosterol forms an orange–red compound with an absorption peak at 440 mμ. The full colour development takes longer than for cholesterol and requires 35 min. to go to completion. It is stable for approximately half an hour. As faecal lipids contain interfering substances that must be removed, it was necessary to carry out the determinations on the digitonides.

For the determination of total sterols a saponification procedure becomes necessary and the method used was that described by Sperry & Webb (1950) with slight modifications.

EXPERIMENTAL AND RESULTS

Reagents and standard solutions

Solvents. Acetone (AnalaR); ethanol–water (1:1, v/v); acetic acid (Merck); aq. 30% (v/v) acetic acid; concentrated sulphuric acid (AnalaR); ethanolic 0·5N-KOH; 1% digitonin in ethanol–water (1:1, v/v) (stored in the dark); $FeCl_3,6H_2O$ (AnalaR) in acetic acid (70 mg./100 ml.; best prepared by dissolving an accurately weighed amount in the required volume of acetic acid. It is unstable in storage and must be prepared daily).

Cholesterol standard. Cholesterol, 0·100 mg./ml. (m.p. 147·5°, recrystallized from light petroleum, b.p. 50–60°), in acetone.

Coprosterol standard. Coprosterol, 0·200 mg./ml. (m.p. 101°; recrystallized from acetone at −10°), in acetone.

Procedures

Free sterols. The lipids are obtained from the minced tissues by two 8 hr. extractions with acetone (5 vol.) followed by one 24 hr. extraction with methanol–light petroleum (b.p. 50–60°) (1:10, v/v) in a Soxhlet extractor.

A portion (1 ml.) of acetone solution containing approx. 1 mg. of lipids is pipetted into a 15 ml. centrifuge tube and digitonin solution (2 ml.) is added. The mixture is stirred vigorously and warmed briefly in the water bath to aid flocculation of the precipitate. The tube is allowed to stand for 15 min. with occasional stirring. The solution is then centrifuged for 10 min. at 2500–3000 *g* and the liquid decanted. Ethanol–water (3 ml.; 1:1, v/v) is added and the precipitate dispersed thoroughly with a glass rod. It is then brought to the boil and allowed to cool for 10 min. This procedure removes occluded digitonin. After cooling, the tube is centrifuged as before, the liquid decanted and the tube drained upside down on a filter paper for 15 min. The precipitate is then dissolved in 3 ml. of $FeCl_3$ reagent at room temperature, as the coprosterol reaction is heat-sensitive. Complete solution is rapidly attained by vigorous stirring. Concentrated sulphuric acid (2 ml.) is then run down the side of the tube so as to form a bottom layer and the whole is finally mixed thoroughly and allowed to stand for 35 min. for the colour to develop fully. A blank, containing $FeCl_3$ reagent and H_2SO_4, is also prepared. The extinction (*E*) is measured, 35–45 min. after the addition of H_2SO_4, at 440 and 560 mμ in a suitable spectrophotometer (e.g. a Beckman model DU) in a 1 cm. cell. The weights of sterols are calculated as described below. It is practicable to carry out six to eight determinations simultaneously.

Total sterols. Sample (1 ml.) is pipetted into a centrifuge tube and the solvent largely removed by evaporation on a water bath. Ethanolic 0·5N-KOH (1 ml.) is added and the sample stirred with a glass rod until the lipids are completely dissolved. The tube is then immersed in a water bath at 38° for 30 min. At the end of this period the solution is neutralized to phenolphthalein with 30% acetic acid, 4–5 drops being required. The contents of the tube are then evaporated almost to dryness and taken up in 1 ml. of acetone. The solution may remain cloudy, owing to the presence of potassium salts, but these are subsequently removed when the digitonides are washed with ethanol–water (1:1, v/v). The determination is then continued as described for free sterols.

Standardization

Varying weights of cholesterol and coprosterol showed that digitonides obey Beer's law at 440 mμ and 440 and 560 mμ respectively. For the preparation of calibration curves solutions containing 0·050, 0·100, 0·150 and 0·200 mg. of cholesterol and 0·20, 0·40, 0·60, 0·80 and 1·00 mg. of coprosterol were evaporated to dryness in

15 ml. centrifuge tubes, redissolved in 1 ml. of acetone and treated as described for free sterols. The extinctions ($E_{1\,cm.}$) were measured at 440 and 560 mμ and plotted against the weights. The mean values of $E_{1\,cm.}$ for 0·10 mg. were found to be 0·3830 and 0·5980 for cholesterol and 0·1237 and 0·0867 for coprosterol at 440 and 560 mμ respectively.

Calculation. The equations used to calculate the weights of cholesterol and coprosterol are derived as follows:

x = cholesterol present (mg.);
y = coprosterol present (mg.).

Then
$$E_{560} = 5{\cdot}98x + 0{\cdot}867y,$$
$$E_{440} = 3{\cdot}83x + 1{\cdot}237y.$$

Therefore
$$x = 0{\cdot}304\,E_{560} - 0{\cdot}2126\,E_{440},$$
$$y = 1{\cdot}467\,E_{440} - 0{\cdot}940\,E_{560}.$$

Accuracy. The method was tested on mixtures of known composition and the recoveries were calculated. The mean recovery was 100·4% (S.D. ±2·80%) for cholesterol and 99·8% (S.D. ±3·41%) for coprosterol.

Interference by other faecal steroids

The method was also tested on faecal lipids from rats on a basal fat-free diet with a 10% corn-oil supplement. The absorption spectrum of this sample, treated as described above, is shown in Fig. 1. It is noted that in this mixture of cholesterol and coprosterol there is a peak at 550 mμ. This is due to a sharp fall in the extinction due to coprosterol above this wavelength, thus causing a decline in the combined extinctions at 560 mμ. Also shown is the spectrum of a synthetic mixture containing the calculated quantities of cholesterol and coprosterol (0·056 and 0·433 mg. respectively).

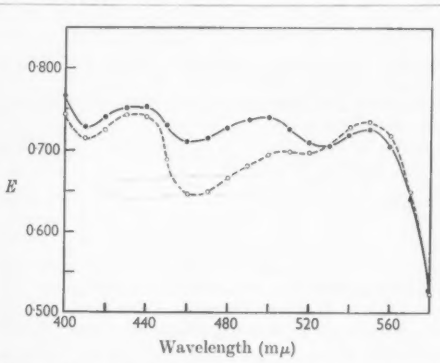

Fig. 1. Absorption spectra of faecal fat from rats on a basal fat-free diet with 10% corn-oil supplement (●) and of a synthetic mixture of the calculated amounts of cholesterol and coprosterol present (○).

Table 1. *Molecular extinction coefficient of the principal faecal sterols*

Substance	ε 440 mμ	ε 560 mμ
Cholesterol	7295	11580
Coprosterol	2386	1610
Δ^7-Cholestenol	0	0
7-Dehydrocholesterol	689	249
Cholestanol	1961	967
β-Sitosterol	5991	8064

The method was further tested for interference by other faecal sterols, namely Δ^7-cholestenol, 7-dehydrocholesterol and cholestanol as well as β-sitosterol, which may be introduced with the diet. The molecular extinctions of these sterols were determined by the above-described method (Table 1). The proportion of the total extinction was calculated for each sterol, based on the sterol composition found by Coleman *et al.* (1956). At 440 mμ it was found that cholesterol and coprosterol accounted for 96·9% of the total extinction, 7-dehydrocholesterol 0·1% and cholestanol 3·0%. At 560 mμ cholesterol and coprosterol contributed 98·3% and cholestanol 1·7% of the total extinction. The so-called 'sterol D' mentioned by these authors could not be tested.

It was found that 7-dehydrocholesterol was poorly precipitated and Δ^7-cholestenol not at all.

DISCUSSION

Although the method described above is based on that described by Zak *et al.* (1957) for the determination of serum cholesterol, a number of modifications, apart from the extension to coprosterol, have been made. Thus the determination of total as well as of free sterol is carried out via digitonide precipitation, a step necessary to eliminate interfering impurities. It appears advisable to incorporate this step into all determinations made by the method of Zak *et al.* (1957), since the present author's experience with depot fats, as well as faecal lipids, supports the conclusions reached by Rhodes (1959) that non-steroid impurities interfere with the method. In the determination of plasma cholesterol Zak *et al.* (1957) used 0·5 ml. of 1% digitonin solution. However, the weights of sterol determined in this procedure are considerably larger, and it was found necessary to increase the amount of digitonin used in order to maintain an adequate excess. The use of 2 ml. of digitonin solution produces a mixture of solvents consisting of equal parts of acetone, ethanol and water, in which the precipitation was carried out. The digitonide was brought to the boil in ethanol–water (1:1) to rid it of occluded digitonin. Digitonin gives a pale-orange compound with considerable absorption at 440 mμ when treated with ferric chloride reagent.

Since the method involves a difference of extinctions it is important to select the weight of sample so that the extinctions of neither sterol at 440 mμ is too low. The method is not considered suitable for the determination of less than 0·025 mg. of cholesterol and 0·075 mg. of coprosterol. Furthermore, it was found that optimum values for E_{440} and E_{560} are between 0·450 and 1·100. The sample quoted represents a typical determination, where 0·8765 mg. of lipid, containing 6·39 % of cholesterol and 49·40 % of coprosterol, had values of E_{440} 0·750 and E_{560} 0·707.

The difference in the spectra also reveals the presence of an unknown impurity with a peak at approx. 460 mμ, resulting in errors of 0·01 and 0·02 mg. for cholesterol and coprosterol respectively. Tests carried out on other faecal sterols (Table 1) show errors of 3·1 and 1·7 % for E_{440} and E_{560} respectively. With the faecal fat just described (fat-free diet with corn-oil supplement) the corrected extinctions should therefore be E_{440} 0·727 and E_{560} 0·695, and the recalculated proportions are therefore cholesterol 6·41 % and coprosterol 47·01 %, thus giving estimated errors of 0·31 % for cholesterol and 4·8 % for coprosterol. These seem to be almost solely attributable to cholestanol, which would appear in the determination chiefly as coprosterol.

Table 1 shows that the molecular extinction of β-sitosterol is relatively high. The effect of this on the accuracy of the method in cases where this compound is part of the diet is difficult to assess. Assuming the ingestion of 2 g. of corn oil (i.e. approx. 10 %) daily, the β-sitosterol intake would amount to 10 mg. From the work of Gould (1955), who estimated β-sitosterol absorption in the intestine to be one-tenth of that of cholesterol, and from the work of Cook & Thomson (1951) on intestinal absorption of cholesterol, it is estimated that rats could absorb 12 mg. of β-sitosterol/day. Furthermore Coleman & Baumann (1957) produced evidence that approx. 70 % of the β-sitosterol is hydrogenated in the intestine. These considerations make it appear likely that β-sitosterol will not interfere appreciably with the method unless the amounts administered are greatly in excess of those quoted above. Nevertheless, the possibility of error, particularly when faecal components other than those mentioned above are present, cannot be ruled out and the value of the method must then be reassessed.

SUMMARY

1. A method based on that of Zak *et al.* (1957) is described for the simultaneous quantitative determination of cholesterol and coprosterol, both free and total, in faecal lipids.

2. The method was tested on mixtures of known composition and it was found that the standard deviations, based on percentage recovery, were ±2·80 and ±3·41 % for cholesterol and coprosterol respectively, and the mean percentage recoveries were 100·4 and 99·8 %.

3. Tests carried out on faecal lipids indicate that other sterols normally present in faeces do not materially affect the results.

4. The method is suitable for determinations on batches of six to eight samples.

REFERENCES

Brown, H. H., Zlatkis, A., Zak, B. & Boyle, A. J. (1954). *Analyt. Chem.* **26**, 397.

Coleman, D. L. & Baumann, C. A. (1957). *Arch. Biochem. Biophys.* **72**, 219.

Coleman, D. L., Wells, W. W. & Baumann, C. A. (1956). *Arch. Biochem. Biophys.* **60**, 412.

Cook, R. P. & Thomson, R. O. (1951). *Quart. J. exp. Physiol.* **36**, 61.

Gould, G. (1955). *Ann. N.Y. Acad. Sci.* (2), **18**, 129.

Rhodes, D. N. (1959). *Biochem. J.* **71**, 26P.

Snog-Kjaer, A., Prange, I. & Dam, H. (1956). *J. gen. Microbiol.* **14**, 256.

Sperry, W. M. & Webb, M. (1950). *J. biol. Chem.* **187**, 97.

Zak, B., Luz, D. A. & Fisher, M. (1957). *Amer. J. med. Tech.* **23**, 283.

Biochem. J. (1960) **77**, 448

5-Fluorouracil and Mucopeptide Biosynthesis by *Staphylococcus aureus*

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The accumulation of uridine mucopeptides has been shown to occur when some strains of *Staphylococcus aureus* are treated with penicillin (Park, 1952*a–c*; Park & Strominger, 1957; Strominger, 1957). In the strain examined, the most complicated of these compounds contained uridine diphosphate linked to one molecule each of 3-carboxyethylglucosamine (muramic acid), D-glutamic acid and L-lysine together with three molecules of alanine; two of the three alanine molecules were in

the D-configuration (Strominger & Threnn, 1959*a*). It has been suggested (Park & Strominger, 1957) that these compounds are precursors of the cell-wall mucopeptides. Other evidence (Park, 1958; Mandelstam & Rogers, 1959) shows that penicillin and some other antibiotics inhibit the synthesis of cell-wall mucopeptides and may therefore be expected to lead to the accumulation of precursors. Uridine mucopeptides also accumulate when cell-wall formation is prevented by the absence of an essential amino acid. For example, when a strain of staphylococcus is incubated in the absence of lysine a mucopeptide containing no lysine accumulates (Strominger & Threnn, 1959*b*). The present work has been designed to examine further, by the use of uridine analogues, the hypothesis that uridine compounds are precursors of the cell-wall mucopeptides. The results of this work are also interesting in relation to the possible mode of action of penicillin.

Duschinsky, Pleven & Heidelberger (1957) first synthesized 5-fluorouracil as a tumour-inhibiting compound. Low concentrations of it were also found (Scheiner, Kostelak & Duschinsky, 1957; Heidelberger *et al.* 1957) to inhibit the growth of a variety of bacteria. It has since been shown that 5-fluorouracil is incorporated into the ribonucleic acids of tobacco mosaic virus (Gordon & Stachlin, 1958), of mouse liver, spleen and tumour (Chaudhuri, Montage & Heidelberger, 1958) and of *Escherichia coli* (Horowitz & Chargaff, 1959). It is also converted into 5-fluorouridine mono-, di- and tri-phosphate together, probably, with 5-fluorouridine diphosphoglucose, although the last-named compound has not been positively identified (Chaudhuri *et al.* 1958). These reactions of the fluorinated compound have been summarized by Harbers, Chaudhuri & Heidelberger (1959) and by Mandel (1959). Ribosidation and ribotidation of fluorouracil have been achieved in a cell-free system (Sköld, 1958). The incorporation of 5-fluorouridylic acid blocks the incorporation of uracil into ribonucleic acid (Melnick, Cantarow & Paschkis, 1958) but allows a net increase of this nucleic acid to occur (Cohen, Flaks, Barner, Loeb & Lichtenstein, 1958). The incorporation of orotic acid into the thymine of deoxyribonucleic acid, on the other hand, is almost completely stopped (Edinoff, Knoll & Klein, 1957), and in *E. coli* 5-fluorouracil and its ribotide completely prevent deoxyribonucleic acid synthesis. These effects on deoxyribonucleic acid formation can be reversed by thymine and thymidine, which also allow growth to occur in the presence of 5-fluorouracil.

In the present work it is the first of the above functions of the 5-fluoro compound, as an analogue of uracil, and not the second, as an inhibitor of thymine synthesis, which is studied. Hence the present interest is in its incorporation into various compounds in place of uridine, rather than in the simultaneous inhibition of thymine and deoxyribonucleic acid formation which also occurs. A preliminary report of some of the earlier stages of this work has already appeared (Rogers & Perkins, 1960) and a report of preliminary investigations with *E. coli* has been published (Tomasz & Borek, 1960).

MATERIALS AND METHODS

Organisms. Staphylococcus aureus strain 524/SC (Rogers, 1953) was used for most of the work but some experiments were also done with strain Oxford. Both strains were maintained as dried cultures.

Growth of bacteria and preparation of cell suspensions. These were as described by Mandelstam & Rogers (1959).

Chemicals. Amino acids labelled at C-1 with ^{14}C were supplied by The Radiochemical Centre, Amersham, and diluted with unlabelled material so that 1 mg. contained 1 μc. These were then dissolved to give stock solutions of 1 mg./ml. 5-Fluorouracil and 5-fluoro[2-^{14}C]uracil were supplied through the courtesy of Dr Morrison of Roche Products Ltd., Welwyn Garden City; 6-azauracil was from California Research Inc.; 6-azathymine was from L. Light and Co. Ltd. and 8-azaguanine from Sigma Chemical Co.

Cell-wall biosynthesis. This was measured by the incorporation of labelled amino acids according to the techniques described by Mandelstam & Rogers (1959). All four amino acids present in the cell-wall mucopeptide (i.e. glutamic acid, glycine, lysine and alanine) were always present; when necessary one was supplemented with the labelled compound. Chloramphenicol (50 μg./ml.) was always present. Likewise the preparations of hot-trichloroacetic acid precipitates and cell walls from the organisms were as described by Mandelstam & Rogers (1959).

Soluble N-acetylhexosamine-containing compounds in the cells. These were extracted with trichloroacetic acid and their content of bound *N*-acetylhexosamine was measured according to Strominger (1957) including the brief preliminary period of hydrolysis with HCl.

Amino acids, glucosamine and muramic acid. These were measured as before (Perkins & Rogers, 1959).

Phosphorus. This was determined by the method of Allen (1940).

Paper chromatography

Samples to be examined by paper chromatography for purine and pyrimidine bases were first hydrolysed in 88% (v/v) formic acid at 165° for 1 hr. The formic acid was then removed *in vacuo*.

Amino acids and amino sugars. The following solvent systems were used: (*a*) butan-1-ol–acetic acid–water (63:10:27), upper phase; (*b*) butan-1-ol–pyridine–water (6:4:3) (Jeanes, Wise & Dimler, 1951).

Nucleotides and purine and pyrimidine bases. The following solvents were used: (*a*) butan-1-ol–ammonia (MacNutt, 1952); (*b*) *iso*propanol–HCl (Wyatt, 1951); (*c*) *iso*butyric acid–ammonia (Zetterström & Ljunggren, 1951); (*d*) ammonium acetate–ethanol, pH 7 and pH 3·8 (Paladini & Leloir, 1952).

Column chromatography

Dowex-1 (× 2, *chloride form*, 50–100 *mesh*). This material was first treated with 2N-NaOH until no further chloride was present in the effluent, washed with water, treated with 2N-HCl until no sodium (by flame test) was eluted and then washed with water until the effluent was chloride free. The samples were applied in about 20 ml. of solution to a column of the resin (15–20 cm. long and 1·5 cm. in diameter) and allowed to flow at a rate of 0·5 ml./min. The column was then washed at the same rate with about 20 ml. of water. After this the first solvent was applied (0·002N-HCl). This and the succeeding solvents were those used by Strominger (1957) to fractionate the compounds, containing hexosamine, accumulated by staphylococci when they are acted upon by penicillin. The rate of flow was maintained at 0·5 ml./min. and 5·0 ml. fractions were collected. The whole operation was conducted at 0–4°. All samples thought to contain material of interest were neutralized to pH 7·0 with N-NaOH and not allowed to stand at an acid pH for longer than 3 hr.

NN-*Diethylaminoethylcellulose*. This was obtained from Kodak Ltd., Kirkby, Lancs. It was first suspended in water and the finer material carefully removed. This is most important if reasonable flow rates in columns are to be achieved. The larger material was then suspended in 0·01 M-ammonium acetate, pH 6·5, and N-acetic acid added drop by drop until the pH was restored to 6·5. A glass column about 10 cm. × 1·5 cm. with a no. 3 sintered disk at the bottom and a little glass wool above it was then packed to give a column about 7 cm. high. This was washed with 0·01 M-ammonium acetate at pH 6·5 until the inflowing and outflowing liquid had the same pH. The sample, dissolved in about 10 ml. of cold 0·01 M-ammonium acetate, pH 6·5, was then applied and allowed to flow through the column at about 0·5 ml./min. After the column was washed, a gradient of ammonium acetate was applied by running a 0·5 M-solution of the salt (for the expt. in Fig. 6 a M-solution was used), adjusted to pH 6·5, into 1 l. of 0·01 M-solution at the same pH contained in a flask fitted with a magnetic stirrer. Fractions of 5 ml. were collected at a flow rate of 0·5 ml./min. and the whole operation was conducted at 0–4°.

The extinction of all the samples coming from both the Dowex-1 and diethylaminoethylcellulose (DEAE) columns was measured in 1 cm. cells at a wavelength of 260 mμ in a Unicam SP. 500 spectrophotometer. A sample (usually 0·4 ml.) of each alternate tube was briefly hydrolysed (Strominger, 1957) and the *N*-acetylhexosamine content measured. In acidifying these samples for hydrolysis, it was necessary to increase the amount of acid added to allow for the increasing concentration of ammonium acetate buffer in the samples. In peaks containing amino sugar compounds every tube was estimated. In the experiments in which 5-fluoro[2-^{14}C]uracil was used 0·1 ml. of each alternate tube was plated and its radioactivity measured; again a sample from each tube was measured in the regions of activity.

Radioactive-counting technique. Sufficient of the dry solid materials (i.e. precipitates, insoluble in hot trichloroacetic acid, and cell walls) was packed in 0·3 cm.^{2} planchets to allow the assumption of an infinitely thick layer. A thousand counts or more above background count were counted, giving an error of ±5%. For samples from chromatograms of which 0·1 ml. was plated on detergent-treated metal planchets to give an infinitely thin layer, counting was not continued until 1000 counts above background had been registered unless the sample was likely to be of subsequent importance (e.g. in the region of the peaks). The count registered in 15 min. was usually recorded. This latter procedure does not allow accurate assessment of any weakly active samples but this sacrifice was necessary where several hundred samples were to be counted. The efficiency of the ordinary end-window counter and scaling apparatus used for this work was such that a standard 1 cm.^{2} polythene disk containing 1 μc/g. gave 960 counts/min.

RESULTS

Inhibition of mucopeptide synthesis

Fig. 1 shows the effect of various concentrations of 5-fluorouracil upon the incorporation of [1-^{14}C]-glutamic acid into the material, insoluble in hot trichloroacetic acid, prepared from cells which had been incubated for 100 min. in the usual system. Incorporation by both strains (524/SC and Oxford) is inhibited, although the effect on 524/SC is greater than that on strain Oxford. A considerable number of experiments of this type have been done, particularly with 524/SC, and the effect of 0·1 μmole of 5-fluorouracil/ml. has been found to vary from about 25 to 60%. Attempts to prevent this variation by first starving the cells of intracellular uridine compounds by preincubating suspensions in salt solutions alone, salts and glucose, or salts and glucose and fluorouracil, have not been successful. The results of the experiments with strain Oxford seemed less variable although the inhibition was not as great.

Previous results (Mandelstam & Rogers, 1959) with strain 524/SC treated with penicillin showed that cell-wall synthesis was at first partially inhibited and only completely stopped after incuba-

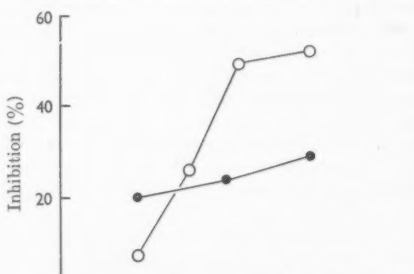

Fig. 1. Effect of various concentrations of 5-fluorouracil upon the incorporation of [1-^{14}C]glutamic acid into the material insoluble in hot trichloroacetic acid. The radioactivity of the materials was measured at infinite thickness. ○, Strain 524/SC; ●, strain Oxford.

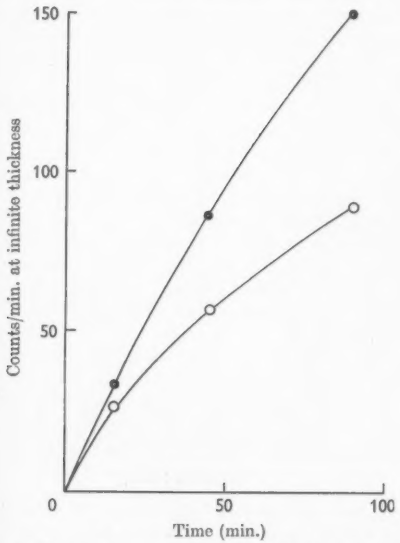

Fig. 2. Rate of incorporation of [1-^{14}C]glutamic acid by strain 524/SC into the material insoluble in hot trichloroacetic acid in the presence (○) and absence (●) of 0·06 μ-mole of 5-fluorouracil/ml.

tion for about 45 min. Fig. 2 shows the effect of 0·06 μmole of 5-fluorouracil/ml. upon the course of incorporation of [1-^{14}C]glutamic acid into the material from cells of 524/SC, which is insoluble in hot trichloroacetic acid. Although there may be some tendency for synthesis in the presence of fluorouracil to decrease more rapidly with time than it does in the control cells, there is no very dramatic difference between the two curves in this respect. 5-Fluorouracil appears to be almost fully effective in the inhibition of synthesis from the start of the experiment. Examination of the effect of fluorouracil upon the incorporation of the four cell-wall amino acids shows (Table 1) that they are all affected, although to slightly different extents. Examination of the composition of the separated cell walls before and after the incubation in the presence of 0·4 μmole of 5-fluorouracil/ml. showed no difference in the molar proportions of the amino acids and amino sugars. Previously, different molar ratios had been found in the mucopeptides from penicillin-treated cells of strain 524/SC (Mandelstam & Rogers, 1959).

Work described above has been done with material insoluble in the hot trichloroacetic acid rather than with isolated cell walls since it was shown earlier (Mandelstam & Rogers, 1958, 1959) that when *Staphylococcus aureus* strain 524/SC is incubated in the presence of chloramphenicol and labelled

Table 1. *Effect of 5-fluorouracil upon the incorporation of the four mucopeptide amino acids into the material insoluble in hot trichloroacetic acid from* Staphylococcus aureus *strain* 524/SC

The washed cells (1 mg./ml.) were incubated with 200 μg./ml. each of alanine, lysine, glycine and glutamic acid, 1% (w/v) glucose, 50 μg. of chloramphenicol/ml. and 0·05 M-potassium phosphate buffer, pH 7·0. The amount of labelled amino acid added was equal to 0·02 μc/ml. Incubation was for 90 min. at 35° with shaking to aerate, and 25 ml. of the suspensions were contained in 100 ml. conical flasks. At the end of the incubation period the flasks were cooled, 2·5 ml. of 50% (w/v) trichloroacetic acid was added to each flask. The resulting precipitates were washed with the appropriate unlabelled amino acid and treated with 5% (w/v) trichloroacetic acid at 90° as described by Mandelstam & Rogers (1959).

Amino acid labelled	Concn. of 5-fluorouracil (μmole/ml.)	Counts/min. at infinite thickness	Inhibition (%)
Glutamic acid	0	144·7	—
	0·05	113·8	22·0
	0·50	94·7	34·5
Glycine	0	1029	—
	0·05	861	17·0
	0·50	749	26·2
Lysine	0	60·2	—
	0·05	53·2	11·9
	0·50	48·3	19·9
Alanine	0	481	—
	0·05	419	13·0
	0·50	344	28·8

Table 2. *Effect of 5-fluorouracil upon the incorporation of* [1-^{14}C]*glutamic acid into material, insoluble in hot trichloroacetic acid, and into cell walls of* Staphylococcus aureus *strain* 524/SC

The conditions of incubation and preparation of the material insoluble in hot trichloroacetic acid were as in the legend to Table 1 except that incubation was continued for 2 hr. The concentration of 5-fluorouracil was 0·4 μmole/ml. The cell walls were prepared from a separate sample of the incubated suspension by the method of Cummins & Harris (1956).

5-Fluorouracil	Counts/min. at infinite thickness: Material insoluble in hot trichloroacetic acid	Counts/min. at infinite thickness: Cell walls
Absent	374	880
Present	180	564
Inhibition by 5-fluorouracil (%)	52	36

cell-wall amino acids, 95% of the incorporated activity appears as the corresponding amino acid in the cell wall, the remaining 5% of activity being present in soluble material from the cell, presumably protein. Therefore, any inhibitory effect of fluorouracil on the 5% of the amino acid which is incorporated into protein is unlikely to influence the results. Table 2 shows that the major part of the

effect of fluorouracil in the system is upon cell-wall synthesis. In this experiment cell walls were separated by the technique of Cummins & Harris (1956). The degree of inhibition of incorporation by 5-fluorouracil is less when measured in isolated cell walls than in material insoluble in hot trichloroacetic acid. The reason for this is not known, but the results differ by more than the maximum total experimental error of the test, which is of the order of 10%. However, it is clear that cell-wall synthesis is inhibited.

Prevention of inhibition. Inhibition of cell-wall synthesis by 5-fluorouracil could be prevented by the simultaneous presence of either uracil or uridine. Both of these compounds were about equally effective on a molar basis. For example, inhibition by 0·06 μmole of 5-fluorouracil/ml. was 50% reversed by an equimolar amount of either uracil or uridine. In a single test 0·06 μmole of thymine/ml. was completely without effect.

Other analogues. The effect of other purine and pyrimidine analogues on strain 524/SC was then tested in the usual systems and again the incorporation of [1-^{14}C]glutamic acid into the material, insoluble in hot trichloroacetic acid, was followed. 8-Azathymine, 8-azaguanine, 6-azauracil and 6-azauracil ribotide were examined, each at an arbitrary concentration of 20 μg./ml. and compared with 5-fluorouracil at concentrations of 15·5 and 2·6 μg./ml. Whereas the latter compound inhibited cell-wall synthesis to the extent of 38 and 15% at these two concentrations, none of the other compounds showed any inhibition of amino acid incorporation. To the extent of this examination the effect of 5-fluorouracil can be said to be specific.

Accumulation of compounds containing amino sugar in inhibited cells

Cells of *S. aureus*, strain 524/SC, were incubated in the usual system with and without 0·4 μmole of 5-fluorouracil/ml. Samples of the suspension were withdrawn at the times indicated in Fig. 3 and cooled. The cells were centrifuged, washed with an equal volume of cold water and then extracted at 0° with cold 10% trichloroacetic acid (1 ml. of acid/20 mg. of cells, for 1 hr.). After this, the insoluble material was deposited by centrifuging and washed. The extract and washings were freed from trichloroacetic acid by ether extraction and the ether was removed in a stream of air.

Samples of the extract were made up to a known volume and estimated for *N*-acetylhexosamine (cf. Methods section). Fig. 3 shows that a very large increase in the soluble compounds containing amino sugar occurred in the presence of 5-fluorouracil. The effect of increasing concentration of the fluoro compound upon the accumulation process is shown in Table 3. These results were obtained after

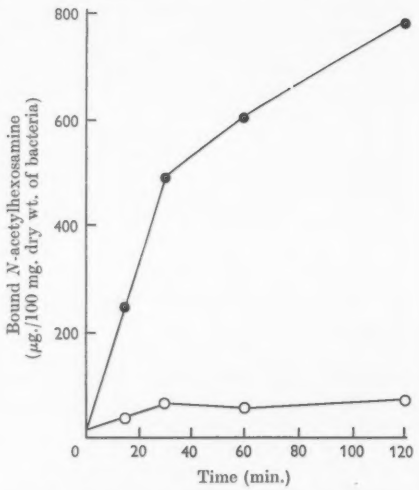

Fig. 3. Rate of formation of material, soluble in trichloroacetic acid and containing bound *N*-acetylhexosamine, by strain 524/SC incubated in the presence (●) and absence (○) of 0·4 μmole of 5-fluorouracil/ml.

Table 3. *Effect of increasing concentration of 5-fluorouracil upon the accumulation of trichloroacetic acid-soluble compounds containing bound* N*-acetylhexosamine in* Staphylococcus aureus *strain* 524/SC

The suspensions were incubated under the conditions shown in the legend to Table 1 for 1 hr. At the end of this period the suspensions were cooled to 0°, and centrifuged. The cells from 25 ml. were then washed once with 10 ml. of ice-cold water, mixed with 1·0 ml. of ice-cold 10% (w/v) trichloroacetic acid and stood at 0–4° for 1 hr. At the end of this period the tubes were centrifuged, the precipitates resuspended in 0·5 ml. of cold 10% (w/v) trichloroacetic acid and centrifuged again. The supernatant solutions and washings were combined, extracted three times with 3 ml. of ethyl ether and the remaining ether was removed in a stream of warm air. The samples were then adjusted to 2·0 ml. and *N*-acetylhexosamine was estimated by the technique of Reissig, Strominger & Leloir (1955) after hydrolysis as suggested by Strominger (1957). The acid used for hydrolysis was neutralized with about 0·05 ml. of N-NaOH with phenolphthalein as internal indicator before the estimation.

Concn. of 5-fluorouracil (μmole/ml.)	Concn. of *N*-acetylhexosamine* (μg./100 mg. dry wt. of cells)
0	26·4
4×10^{-3}	60·0
4×10^{-2}	142·0
2×10^{-1}	432·0
4×10^{-1}	506·4

* The results are expressed in terms of *N*-acetylglucosamine.

incubation for 1 hr. which is after the accumulation process has ceased to occur at a maximum rate with the higher concentrations of inhibitor (see Fig. 3). It was necessary, however, to use this longer time, rather than the ideal of about 20–30 min. in order to obtain sufficiently large amounts of *N*-acetylhexosamine to make estimations accurate at the lower concentrations of fluorouracil.

Prevention of 5-fluorouracil effect. If uracil or uridine were present together with 5-fluorouracil during the incubation, accumulation of compounds containing *N*-acetylhexosamine was prevented. As is shown in Fig. 4 the riboside is slightly more effective than the free base on a molar basis. The molar ratio of 5-fluorouracil to uracil for 80% prevention of inhibition is about 1:1, whereas for the uridine it is about 0·7. The addition of an equimolar amount of thymidine simultaneously with the 5-fluorouracil caused very much less suppression (35–40%) of the accumulation of soluble compounds containing *N*-acetylamino sugar.

Reversal of 5-fluorouracil effect. The above results show that either uracil or uridine can prevent the accumulation of compounds containing amino sugars which are soluble in trichloroacetic acid when they are present simultaneously with the fluorinated analogue. It remained to be shown whether these compounds, accumulated in the presence of fluorouracil, could be used by the cell for wall-mucopeptide synthesis when the fluoro compound was removed. To test this the cells were first treated with 5-fluorouracil and the compounds containing amino sugar allowed to accumulate; the cells were then removed by centrifuging, washed and incubated with uridine. Only a slight fall occurred in the concentration of the preformed compounds. Similar results were obtained when the pretreated cells were incubated with different combinations of amino acids and glucose (cf. Table 4). Thus it appears that once formed these compounds are not readily used for synthesis. If labelled glycine were included in the second incubation mixture it could be shown that mucopeptide synthesis was proceeding in the cells which had first been treated with 5-fluorouracil, washed and subsequently reincubated in the normal system. Thus the compounds containing amino sugar, which accumulated, did not disappear rapidly even though cell-wall synthesis was going on.

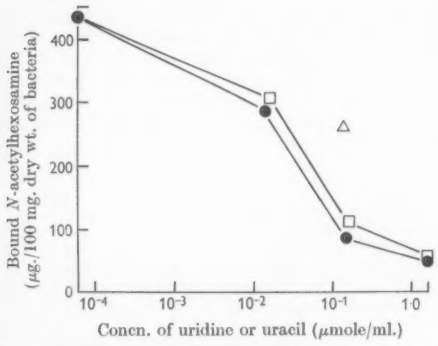

Fig. 4. Prevention of the accumulation of materials, soluble in trichloroacetic acid and containing bound *N*-acetylhexosamine, by uracil (□) and uridine (●). The point at △ represents the test with a single concentration of thymidine. The concentration of 5-fluorouracil was 0·2 μmole/ml. in each test.

Nature of the compounds containing amino sugar

These compounds were allowed to accumulate in larger volumes of suspensions of the strain 524/SC in the presence of 5-fluorouracil and the cells were then extracted with cold trichloroacetic acid in the usual way. The volumes of the extracts obtained were sometimes reduced by evaporation from the frozen state at low pressure after the trichloroacetic acid had been removed by ether extraction and the

Table 4. *Influence of various conditions of reincubation on cells which have already accumulated compounds soluble in trichloroacetic acid and containing amino sugar*

The compounds containing amino sugar were first allowed to accumulate in the cells by incubating a suspension under the usual conditions for 1 hr. at 35° with 0·4 μmole of 5-fluorouracil/ml. These cells were then washed once with ice-cold 0·1 M-potassium phosphate buffer at pH 7·0 and resuspended in the presence of the substances shown below. The four cell-wall amino acids and glucose were at the concentrations shown in the legend to Table 1. Chloramphenicol (50 μg./ml.) and phosphate (0·05 M, pH 7·0) were always present. Uridine was present at a concn. of 0·4 μmole/ml. The experiment was done in duplicate and the suspensions were reincubated at 35° for 1 hr. with shaking.

Conditions of incubation	Bound *N*-acetylhexosamine* (μg./100 mg. dry wt.)	Change from control† (μg./100 mg. dry wt.)
Control (not incubated)	375, 358	—
1. Potassium phosphate buffer (pH 7·0) alone	320‡	−46
2. As (1) + glucose	277‡	−89
3. As (2) + four cell-wall amino acids	338, 318	−36
4. As (3) + uridine	263, 283	−93

* Expressed in terms of *N*-acetylglucosamine.
† Mean values.
‡ Duplicates lost.

pH adjusted to 7·0. The details of these experiments were as follows:

Cell suspensions (1600 ml., 1 mg./ml.) containing the usual four amino acids, glucose, phosphate, 50 μg./ml. of chloramphenicol and 0·1 μmole of 5-fluorouracil/ml. were incubated for 60 min. at 35° in eight 1 l. conical flasks (200 ml./flask) with constant shaking to ensure aeration. At the end of this time the flasks were cooled and the contents centrifuged. The cells were washed and the trichloroacetic acid extract was made in the usual way. In a typical experiment the final volume of the extract, after removal of trichloroacetic acid and ether, was 38 ml. which contained a total of 3·46 mg. of bound amino sugar, estimated as *N*-acetylglucosamine.

This extract was then applied to a Dowex-1 column which was washed and developed as described in the Methods section.

Examination of the samples from the column at a wavelength of 260 mμ showed the presence of several peaks likely to contain nucleotides, but when the bound *N*-acetylhexosamine was measured only two of these peaks contained both types of substance (see Fig. 5). In the particular experiment under consideration peak I contained 49 % of the bound *N*-acetylamino sugar and peak II 35 %, giving a total recovery of 84 % of the bound amino sugar applied to the column. In addition, a further 5–10 % of the compounds containing amino sugars ran through the column with the washings and was presumed to be free *N*-acetylhexosamine. The two peaks containing hexosamine, obtained from the examination of extracts from cells treated with 5-fluorouracil, correspond exactly, in elution position, with the two peaks that Strominger (1957) found in similar extracts from cells treated with penicillin.

The samples from each of the peaks were combined to give two pools, and passed through small carbon–Celite columns (the carbon was Ultra-sorb ZF, British Carbo-Norit Union Ltd., London Road, West Thurrock, Grays, Essex). These columns were then washed with about 100 ml. of water and eluted with 40 % (v/v) aqueous ethanol containing 1 % (v/v) ammonia solution (sp.gr. 0·880) (Cabib, Leloir & Cardini, 1955). The yield of bound amino sugar from this process was never better than about 70 % and often as low as 60 %. However, no better method for concentrating the substances was found. Alcohol and ammonia were removed from these eluates under the vacuum given by a water pump

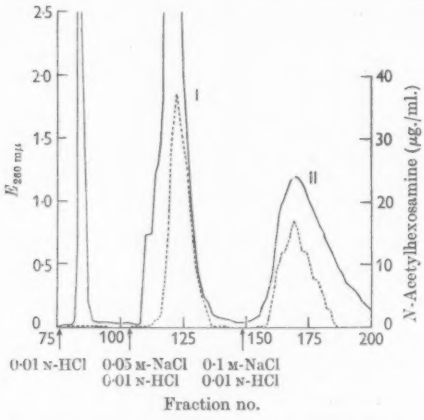

Fig. 5. Elution pattern obtained from a column of Dowex-1 (Cl-form) after application of the trichloroacetic acid extracts from cells of strain 524/SC which had been incubated for 1 hr. with 0·1 μmole of 5-fluorouracil/ml. added to the usual system. ——, Extinction measured at 260 mμ; - - - - -, *N*-acetylhexosamine measured after brief acid hydrolysis of the samples.

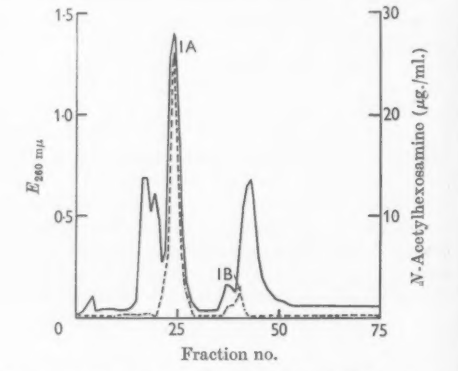

Fig. 6. Elution pattern obtained from DEAE columns when material in peak I (see Fig. 5) from Dowex-1 had been applied. Elution was with a gradient of ammonium acetate at pH 6·5, starting with a concentration of 0·01 M. ——, Extinction at 260 mμ; - - - - -, *N*-acetylhexosamine after brief acid hydrolysis.

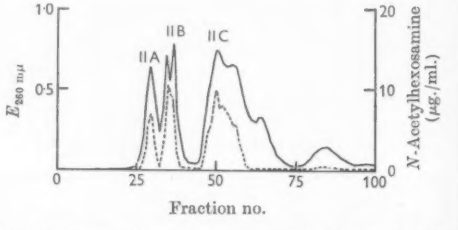

Fig. 7. Elution pattern obtained from DEAE columns when material in peak II (see Fig. 5) had been applied (see legend to Fig. 6 for remaining details).

at a bath temperature of less than 40°. Finally they were dried *in vacuo* over calcium chloride.

Material from each of the dried concentrates was then dissolved and passed down a separate column of DEAE. Elution was with a gradient of ammonium acetate at pH 6·5 (cf. Methods section). Figs. 6 and 7 show that both the peaks from the Dowex-1 separation were complex with respect to nucleotide material but the second peak was more complex with respect to bound amino sugar substances than the first. DEAE columns appeared to be satisfactory for the separation of the substances when developed as described in the Methods section. No experiments, however, were done to ascertain whether or not this substituted cellulose was satisfactory for the separation of nucleotides in general. Korn (1959) has described the use of another cellulose product for nucleotide analysis.

Dowex-1 peak I *material.* About 80% of all the bound hexosamine was contained in a single sharp peak obtained from DEAE (Fig. 6, peak IA). The samples comprising this peak were pooled and dried *in vacuo* during which process the ammonium acetate was removed. Examination of the resulting material by paper chromatography showed a single spot. Samples were then hydrolysed and examined by paper chromatography with separate solvent systems for amino acids, amino sugars and pyrimidine bases. The amino acids present were lysine, glutamic acid and alanine, the amino sugar was muramic acid and the base 5-fluorouracil.

Also present in the DEAE fractions was the very small peak, containing bound hexosamine, at B in Fig. 6. The amount of material here was very small but when hydrolysed by the same methods as those used for the material from peak A, the amino acids recognized were glycine, glutamic acid and alanine, and the amino sugar was muramic acid. The base could not be identified owing to the small amounts of material available. These same two mucopeptides in the 'Dowex-peak 1' material could also be separated by direct paper chromatography in butanol–acetic acid. Consideration of the quantitative data on peak IA from DEAE will be delayed until the experiments with labelled 5-fluorouracil are described.

Dowex-1 peak II *material.* When material from the second peak from Dowex-1 was run on DEAE three peaks, containing both nucleotide material and bound amino sugar, were found. The samples in these peaks were combined to give three pools and the materials they contained were examined as before. Table 5 summarizes the qualitative results obtained. Both uracil and 5-fluorouracil were to be found associated with *N*-acetylglucosamine in the absence of amino acids. Also 5-fluorouracil and uracil were associated with muramic acid and alanine. Estimations showed that this peak IIC, which was clearly still heterogeneous, contained more muramic acid than alanine on a molar basis (see Table 6).

Experiments with 5-fluoro[2-^{14}C]uracil

The labelled 5-fluorouracil as received had a specific activity of 10·6 μc/μmole. This material (1·88 μmoles) was diluted with unlabelled material (400 μmoles) and incubated at a final concentration of 0·4 μmole/ml. in the usual system for studying the biosynthesis of cell wall; strain 524/SC was used. The fluorouracil concentration was four times as high as that in the previous experiments. This change was originally made in order to increase the amount of the compounds, containing bound amino sugar, available for analysis (cf. Table 3). About 20% of the fluorouracil supplied to the organisms was in the trichloroacetic acid extracts from the cells and 50–60% remained in the supernatant solutions prepared from the incubated suspensions. The remaining 20–30% was presumably mostly incorporated into the ribonucleic acid of the cells although this was not examined.

The trichloroacetic acid extracts were treated and analysed exactly as before. The distribution of radioactivity due to 5-fluorouracil, bound amino sugar and material absorbing light of 260 mμ wavelength in the samples from the Dowex-1 column was examined. Three peaks containing radioactivity appeared. The first accounted for about

Table 5. *Components recognized as present in the compounds in the peaks from the diethylaminoethylcellulose columns*

The columns were run and the samples hydrolysed under the conditions given in the Methods section. The symbol (–) means not present.

Dowex peak	DEAE peak	Amino acid: Glutamic acid	Glycine	Lysine	Alanine	Amino sugar	Base
I	A	+	–	+	+	Muramic acid	Fluorouracil
	B	+	+	–	+	Muramic acid	Not identified
II	A	–	–	–	–	Glucosamine	Uracil
	B	–	–	–	–	Glucosamine	Fluorouracil
	C	–	–	–	+	Muramic acid	Fluorouracil, uracil

4–5 % of the applied material but contained no bound amino sugar and was ignored. The second (referred to previously and subsequently as peak I) contained amino sugar and accounted for 53 % of that which had been applied to the column and 31 % of the radioactivity. The third peak containing radioactivity (i.e. the second containing both bound amino sugar and radioactivity, peak II) accounted for 50 % of the applied amino sugar and 46 % of the radioactivity. Hence the total recovery of radioactivity (presumably fluorouracil) was 77 % and of bound amino sugar 103 %. No further solvents were run through the column, in view of these high yields.

The solutions comprising these two peaks were then concentrated by adsorption and elution from carbon as before and applied to columns of DEAE. Figs. 8 and 9 show the results obtained. Both the pictures are more simple than those shown in Figs. 6 and 7. Qualitative examination of IA (Fig. 8) showed that it exactly corresponded to the previous peak IA. Recovery in this peak was 73 % of the radioactivity applied and 98 % of the bound amino sugar. No peak corresponding to IB (Fig. 6) appeared. Peak II (Dowex) material split into two instead of three peaks containing bound amino sugar and nucleotide. The first, called IIB, contained 35 % of the bound amino sugar and IIC, still obviously heterogeneous, contained 48 % giving a total yield of 83 % of the applied material. The corresponding figures for radioactivity were 44 and 37 %.

Thus the use of the higher concentration of fluorouracil had eliminated the material (IIA in Fig. 7) which contained uracil and *N*-acetylglucosamine, leaving that containing the fluorouracil and *N*-acetylglucosamine.

Quantitative examination of the fractions containing muramic acid. Table 6 shows the analysis obtained for the materials isolated from peaks IA and IIC. It will be seen that IA contains fluorouracil, phosphorus, muramic acid, glutamic acid, lysine and alanine in the approximate ratios 1:2:1:1:1:3. Thus the analysis of this compound corresponds to that of 'compound 3' isolated by Park (1952*b*) from staphylococci treated with penicillin with the exception, of course, that uracil is replaced by fluorouracil. This compound is presumably the fluorouridine analogue of the Park (1952*b*) mucopeptide 3. The material from peak IIC contains muramic acid and phosphorus in the molar ratio of approximately 1:2 together with a small proportion of alanine. This was re-examined by the use of a longer (20 cm.) column of DEAE adjusted to pH 5·0 and eluted with a gradient of ammonium acetate buffer (0·01–0·5 M) also at pH 5·0. Two partially separated peaks were obtained containing both fluorouracil and *N*-acetylhexosamine. Ana-

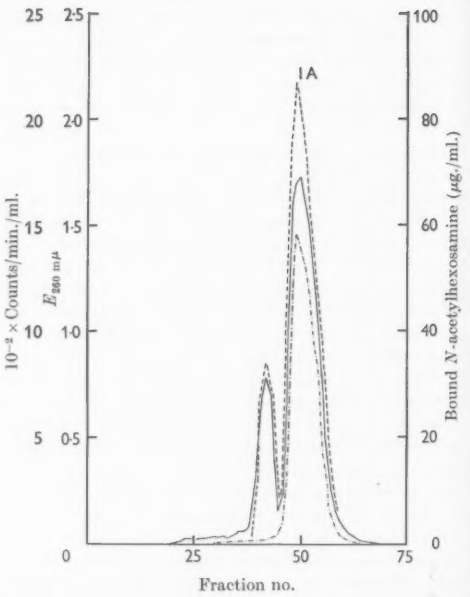

Fig. 8. Elution pattern obtained from DEAE column after trichloroacetic acid extracts from cells of 524/SC treated with 0·4 μmole of labelled 5-fluorouracil/ml. had been passed through Dowex-1 (Cl-form) and the material in peak I (see Fig. 5) isolated. For the method of elution of the DEAE column see the legend to Fig. 6. - - - - -, Radioactivity expressed as counts/min./ml.; ——, extinction at 260 mμ; – · – · –, amounts of *N*-acetylhexosamine/ml., measured after brief acid hydrolysis.

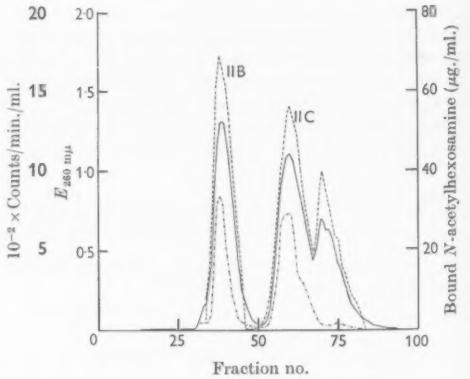

Fig. 9. Elution pattern obtained from DEAE columns after the application of material from peak II from Dowex-1 (cf. Fig. 5). For the remainder of the details of this experiment see the legend to Fig. 8.

Table 6. *Quantitative analysis of fractions, containing muramic acid, isolated by the use of diethylaminoethylcellulose columns from trichloroacetic acid extracts of cells treated with 5-fluoro*[2-^{14}C]*uracil*

Preparation of the samples and the analytical methods are described in the text. The value for fluorouracil is taken as 1·00. The concentration of fluorouracil was estimated from the radioactive count.

	Molar proportion						
Sample	Fluorouracil	P	Glutamic acid	Lysine	Alanine	Muramic acid	Glucosamine
1A	1·00	2·21	0·99	1·05	2·83	1·12	None
2C	1·00	2·24	None	None	0·31	1·04	None

lysis showed that the first of these contained fluorouracil, muramic acid and alanine in the ratio 1·0:1·3:1·0, and the second fluorouracil and muramic acid in the ratio 1·0:1·0, but no alanine. Hence the material in peak IIC is a mixture of fluorouridine diphospho-*N*-acetylmuramic acid and this compound linked to alanine.

The compound containing N-acetylglucosamine. When the material in the peak II (Fig. 5) from the Dowex-1 column was examined by chromatography on the DEAE columns the sharp well-separated peak IIB emerged (cf. Fig. 9). When this material was hydrolysed it was found to contain fluorouracil and glucosamine but no amino acids or muramic acid. These were thought likely to arise from hydrolysis of fluorouridine diphospho-*N*-acetylglucosamine. Examination of the ratios of base:phosphorus:amino sugar, in the concentrated material from peak IIB, however, did not agree with this possibility. Further examination of the individual samples from the column showed that although the ratio of radioactivity to extinction at 260 mμ remained constant, that of radioactivity to *N*-acetylhexosamine rose steeply towards the end of the peak. This suggested that the high ratio of base and phosphorus to amino sugar which had been found in the pooled material might be due to a mixture of a fluorouridine phosphate with fluorouridine diphospho-*N*-acetylglucosamine. In consequence the material from peak IIB from the DEAE column was concentrated and the ammonium acetate was removed by evaporation from the frozen state in the usual way; the material was then dissolved in water and examined by paper chromatography. After being run for 3 days in the *iso*butyric acid–ammonia soln. solvent system, two ultraviolet-absorbing spots appeared, both of which contained radioactivity. Only one of these spots contained *N*-acetylglucosamine and it moved almost exactly the same distance as a marker of uridine diphospho-*N*-acetylglucosamine. This material was eluted with water and its radioactivity and *N*-acetylglucosamine content were measured. The usual brief period of hydrolysis was used to liberate the *N*-acetylglucosamine from the nucleotide. The ratio of base to *N*-acetylamino sugar was 1:0·97 and it seems probable, therefore, that the amino sugar was bound as fluorouridine diphospho-*N*-acetylglucosamine.

DISCUSSION

As with other types of cell, 5-fluorouracil enters staphylococci easily and the internal concentration of further metabolites of the compound becomes considerable. When the cells are supplied with an external concentration of 0·4 μmole of 5-fluorouracil/ml. the total internal concentration of the free and combined compound rises to a calculated value of about 1 μmole/ml. of cell water. Of this material about 3–4 % is not retained by a Dowex-1 column and may be free fluorouracil or its riboside. Of the remainder, 70–80 % is present in combination with amino sugars and amino acids. About half this combined material is as a fluorinated nucleotide–mucopeptide compound which appears to be identical, except for the replacement of uridine by fluorouridine, with the largest of the three compounds isolated by Park (1952*a*, *b*, *c*). Of the remainder, some is present as fluorinated derivatives of fragments of this compound (i.e. fluorouridine diphospho-*N*-acetylmuramic acid and fluorouridine diphospho-*N*-acetylmuramic acid–alanine), and some as fluorouridine diphospho-*N*-acetylglucosamine. In the presence of 0·4 μmole of fluorouracil these appear to be the only compounds, containing both nucleotide and *N*-acetylamino sugars, which accumulate. It seems reasonable to suppose that these are uridine-activated precursors of cell-wall mucopeptide. The normal analogues of these compounds also accumulate in other strains of staphylococci treated with penicillin (Park, 1952*a*, *b*, *c*; Park & Strominger, 1957) or with a unique sample of gentian violet (Strominger, 1959). It was originally proposed that they were the precursors of the cell-wall mucopeptides because the molecular proportions of amino sugars and amino acids in them were the same as those in the cell walls of the organisms examined (Park & Strominger, 1957; Strominger, Park & Thompson, 1959). In the present instance it also seems reasonable to implicate the substances, accumulating in the presence of

5-fluorouracil, as precursors of the cell-wall mucopeptide. In this instance, however, the molecular proportions of the components are quite different from those in the cell-wall mucopeptides of the organisms which accumulate them (Mandelstam & Rogers, 1959; Rogers & Perkins, 1959). In this connexion, the metabolic path of glycine in cells treated with fluorouracil must be considered. Rogers & Perkins (1960) reported that this amino acid is found in small concentrations in hydrolysates of materials from both the peaks obtained from Dowex-1. Nevertheless, it is not a component of any of the compounds isolated when the cells have been treated with the higher concentration of 5-fluorouracil of 0·4 μmole/ml. A definite compound containing glycine, however, can be isolated from cells treated with the lower concentration of 5-fluorouracil of 0·1 μmole/ml. (i.e. the small peak IIB). The nature of the base associated with this material could not be definitely identified owing to the small amount available. It cannot be concluded that the material in this small peak is not an important precursor of the cell-wall mucopeptide because large amounts of it do not accumulate in the presence of 5-fluorouracil. It might be that it is attached to a base other than uracil in which case it would not necessarily be expected to accumulate. This material contained glutamic acid, alanine and muramic acid as well as glycine but it contained no lysine and could be either another precursor of the cell-wall mucopeptide, or the precursor of a second mucopeptide present in the wall. If either of these possibilities is true the molecular proportions of the components of the wall mucopeptide or mucopeptides might be expected to differ from those of the major component accumulating with 5-fluorouracil.

The similarity between the substances accumulating in the presence of 5-fluorouracil and those accumulating in the presence of penicillin is remarkable. The only clear difference, apart from the expected abnormal bases, is the presence of the fluorinated derivative of uridine diphospho-*N*-acetylglucosamine when the cells have been treated with 5-fluorouracil. The normal compound has not been reported to accumulate in the presence of penicillin. One could suppose that penicillin either interfered with the metabolism of uridine diphospho-*N*-acetylmuramic acid compounds or suppressed the formation of uridine diphospho-*N*-acetylglucosamine. Either mechanism would interfere with the formation of the cell-wall mucopeptide. However, uridine diphospho-*N*-acetylglucosamine has been involved in the biosynthesis of mucopolysaccharides such as chitin (Glaser & Brown, 1957) and hyaluronic acid (Markovitz, Cifonelli & Dorfman, 1958, 1959) and these processes are not known to be penicillin sensitive. These facts do not necessarily exclude the possibility that penicillin acts upon the *N*-acetylglucosamine pathway, but the emphasis could be thrown upon peculiarities of the enzymes involved in staphylococci and other penicillin-sensitive micro-organisms, rather than upon the substrates.

The present work, of course, does not suggest that 5-fluorouracil stops the growth of micro-organisms by interfering with cell-wall synthesis. It is much more likely that this happens by inhibition of deoxyribonucleic acid synthesis. It is probable, however, that fragility effects such as those observed (Tomasz & Borek, 1959) in *E. coli* as a result of exposure to the fluorouracil are due to inhibition of cell-wall synthesis, as suggested by Tomasz & Borek (1960). Fluorouracil is not unique among uracil analogues in its effects upon cell-wall synthesis by micro-organisms. For example, Otsuji & Takagi (1959) and Takagi & Otsuji (1958) have shown that when azauracil acts on *E. coli*, bound amino sugars, soluble in trichloroacetic acid, accumulate; these authors did not examine the effects upon mucopeptide formation nor did they fractionate the bound amino sugar compounds. Also Sells (1959) showed that 6-azauracil inhibited cell-wall formation in *Bacillus cereus*. In the present work 6-azauracil or its ribotide led to an accumulation of soluble bound amino sugar although the amounts were small; but at the concentrations used, and under our conditions, these compounds did not inhibit the formation of mucopeptide.

SUMMARY

1. 5-Fluorouracil partially inhibits cell-wall mucopeptide synthesis by *Staphylococcus aureus* strain 524. This inhibition can be prevented by the simultaneous presence of uracil or uridine.

2. During incubation in the presence of 5-fluorouracil, compounds accumulate which contain bound amino sugar and are soluble in trichloroacetic acid; this accumulation is prevented by the simultaneous presence of uracil or uridine.

3. The compounds containing bound amino sugars which have accumulated are not readily utilizable by the cell for mucopeptide synthesis when fluorouracil is removed.

4. Fractionation of the material, soluble in trichloroacetic acid and containing bound amino sugars, has revealed the presence of four compounds: (1) containing fluorouracil, phosphorus, muramic acid, alanine, glutamic acid and lysine with the molecular proportions of 1:2:1:3:1:1; (2) a fluorouridine–phosphorus–*N*-acetylglucosamine compound with molecular proportions of 1:2:1; (3) a mixture of fluorouridine–muramic acid–alanine compounds which has not been fractionated completely but is likely to be fluorouridine

diphospho-*N*-acetylmuramic acid and this compound linked to alanine.

5. The concentration of fluorouracil seems to decide whether or not non-fluorinated uridine compounds of similar composition also accumulate. At higher concentrations of fluorouracil these were not found.

6. The relationship of the accumulation of the fluorouridine compounds to the penicillin-induced formation of the corresponding non-fluorinated compounds is discussed.

REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858
Cabib, E., Leloir, L. F. & Cardini, C. E. (1955). *J. biol. Chem.* **203**, 1055.
Chaudhuri, N. K., Montage, B. J. & Heidelberger, C. (1958). *Cancer Res.* **18**, 318.
Cohen, S. S., Flaks, J. G., Barner, H. D., Loeb, M. R. & Lichtenstein, J. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 1004.
Cummins, C. S. & Harris, H. (1956). *J. gen. Microbiol.* **14**, 583.
Duschinsky, R., Pleven, E. & Heidelberger, C. (1957). *J. Amer. chem. Soc.* **79**, 4559.
Edinoff, M. L., Knoll, J. E. & Klein, D. (1957). *Arch. Biochem. Biophys.* **71**, 274.
Glaser, L. & Brown, D. H. (1957). *J. biol. Chem.* **228**, 729.
Gordon, M. P. & Stachlin, M. (1958). *J. Amer. chem. Soc.* **80**, 2340.
Harbers, E., Chaudhuri, N. K. & Heidelberger, C. (1959). *J. biol. Chem.* **234**, 255.
Heidelberger, C., Chaudhuri, N. K., Danneberg, P., Mooren, D., Griesbach, L., Duschinsky, R., Schnitzer, R. J., Pleven, E. & Scheiner, J. (1957). *Nature, Lond.*, **179**, 663.
Horowitz, J. & Chargaff, E. (1959). *Nature, Lond.*, **184**, 1213.
Jeanes, A., Wise, C. S. & Dimler, R. J. (1951). *Analyt. Chem.* **23**, 415.
Korn, E. (1959). *Biochim. biophys. Acta*, **32**, 554.
MacNutt, W. S. (1952). *Biochem. J.* **50**, 384.
Mandel, H. G. (1959). *Pharmacol. Rev.* **11**, 743.
Mandelstam, J. & Rogers, H. J. (1958). *Nature, Lond.*, **181**, 956.
Mandelstam, J. & Rogers, H. J. (1959). *Biochem. J.* **72**, 654.
Markovitz, A., Cifonelli, J. A. & Dorfman, A. (1958). *Fed. Proc.* **17**, 269.
Markovitz, A., Cifonelli, J. A. & Dorfman, A. (1959). *J. biol. Chem.* **234**, 2343.
Melnick, I., Cantarow, A. & Paschkis, K. E. (1958). *Arch. Biochem. Biophys.* **74**, 281.
Otsuji, N. & Takagi, Y. (1959). *J. Biochem., Tokyo*, **46**, 791.
Paladini, A. C. & Leloir, L. F. (1952). *Biochem. J.* **51**, 426.
Park, J. T. (1952*a*). *J. biol. Chem.* **194**, 877.
Park, J. T. (1952*b*). *J. biol. Chem.* **194**, 885.
Park, J. T. (1952*c*). *J. biol. Chem.* **194**, 897.
Park, J. T. (1958). *Biochem. J.* **70**, 2P.
Park, J. T. & Strominger, J. L. (1957). *Science*, **125**, 99.
Perkins, H. R. & Rogers, H. J. (1959). *Biochem. J.* **72**, 647.
Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955). *J. biol. Chem.* **217**, 959.
Rogers, H. J. (1953). *J. Path. Bact.* **66**, 545.
Rogers, H. J. & Perkins, H. R. (1959). *Nature, Lond.*, **184**, 520.
Rogers, H. J. & Perkins, H. R. (1960). *Biochem. J.* **74**, 6P.
Scheiner, J. M., Kostelak, R. & Duschinsky, R. (1957). *Fed. Proc.* **16**, 242.
Sells, H. (1959). *Biochem. Pharmacol.* **2**, 255.
Sköld, O. (1958). *Biochim. biophys. Acta*, **29**, 651.
Strominger, J. L. (1957). *J. biol. Chem.* **224**, 509.
Strominger, J. L. (1959). *J. biol. Chem.* **234**, 1520.
Strominger, J. L., Park, J. T. & Thompson, R. E. (1959). *J. biol. Chem.* **234**, 3263.
Strominger, J. L. & Threnn, R. H. (1959*a*). *Biochim. biophys. Acta*, **33**, 280.
Strominger, J. L. & Threnn, R. H. (1959*b*). *Biochim. biophys. Acta*, **36**, 83.
Takagi, Y. & Otsuji, N. (1958). *Biochim. biophys. Acta*, **29**, 227.
Tomasz, A. & Borek, E. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 929.
Tomasz, A. & Borek, E. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 324.
Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.
Zetterström, R. & Ljunggren, M. (1951). *Acta chem. scand.* **5**, 291.

Biochem. J. (1960) **77**, 459

A Spectrophotometric Study of the Reaction Catalysed by Serine Transhydroxymethylase

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In previous papers (Blakley, 1958, 1959, 1960) evidence has been presented that formaldehyde reacts non-enzymically and reversibly with tetrahydropteroylglutamic acid. A comparison of the equilibrium constant of this reaction with those obtained from the reaction of formaldehyde with other hydropteridines indicated that N^5N^{10}-methylenetetrahydropteroylglutamate is the product formed from tetrahydropteroylglutamate, although N^5-hydroxymethyltetrahydropteroylglu-

tamate may possibly occur as an intermediate in the reaction.

The synthesis of serine from glycine and formaldehyde, as catalysed by serine transhydroxymethylase, requires tetrahydropteroylglutamate (tetrahydroPGA) as coenzyme, and in view of the non-enzymic reaction between formaldehyde and tetrahydropteroylglutamate it seems reasonable to assume that the reaction proceeds in two steps:

$$\text{TetrahydroPGA} + \text{H·CHO} \rightleftharpoons N^5N^{10}\text{-MethylenetetrahydroPGA} \quad (1)$$

$$N^5N^{10}\text{-MethylenetetrahydroPGA} + CH_2(NH_2)\cdot CO_2H \rightleftharpoons \text{TetrahydroPGA} + CH_2(OH)\cdot CH(NH_2)\cdot CO_2H \quad (2)$$

Evidence in support of this assumption has now been obtained by spectrophotometric methods and the equilibrium constant for reaction (2) has been determined.

MATERIALS AND METHODS

Glucose 6-phosphate (crystalline barium salt), reduced triphosphopyridine nucleotide (89% purity) and glucose 6-phosphate dehydrogenase (activity; 800 μmoles of triphosphopyridine nucleotide reduced/min./g.) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Dihydropteroylglutamic reductase. This was purified from extracts of *Streptococcus faecalis* Rogers (American Type Culture Collection 8043) by an unpublished method. In terms of the units defined by Osborn & Huennekens (1958) the preparation had an activity of 398 units/mg.

NN-Diethylaminoethylcellulose. This was obtained from Eastman Organic Chemicals, New York, and triethylaminoethylcellulose was prepared from it by the method of Porath (1957).

Dihydropteroylglutamic acid. This was prepared by the method of Futterman (1957) from purified pteroylglutamic acid (PGA), and dried at room temperature (0·05 mm. Hg) over P_2O_5 and KOH.

Tetrahydropteroylglutamic acid. The racemic compound was prepared by catalytic hydrogenation of purified PGA in neutral aqueous solution (Blakley, 1957).

The active isomer was generated enzymically in the reaction mixture by the action of dihydropteroylglutamic reductase. Additions to the reaction mixture for this purpose were as follows: dihydropteroylglutamic acid (dihydroPGA; 0·03 mM), reduced triphosphopyridine nucleotide (0·04 mM), 2:3-dimercaptopropanol (5 mM), dihydropteroylglutamic reductase (5·8 units/ml.), glucose 6-phosphate dehydrogenase (0·04 unit/ml.), glucose 6-phosphate (3·3 mM). These components (except dihydroPGA) were also added when DL-tetrahydroPGA was used.

Serine transhydroxymethylase. The enzyme, prepared as described by Blakley (1959), was not sufficiently pure for spectrophotometric studies. Further purification was therefore attempted with ion-exchange chromatography. In preliminary studies with columns of diethylaminoethylcellulose and triethylaminoethylcellulose it was observed that when the column was buffered at pH 8 or lower the serine transhydroxymethylase passed straight through the column, even at low buffer concentrations (e.g. 0·01 M-phosphate), with a three- or four-fold purification. Since this indicated that the enzyme was a basic protein with isoelectric point above pH 8 purification on the cation-exchange resin Amberlite IRC (XE64) was next attempted. The purification procedure finally adopted was as follows.

The enzyme was extracted and purified from the livers of four rabbits as previously described (Blakley, 1959). These preparations, of which many have now been made in this laboratory, range in activity from 40 to 120 μmoles of serine/hr./mg. of protein, measured in the standard assay system (Blakley, 1954), the average activity being about 75 μmoles of serine synthesized/hr./mg. of protein. This enzyme preparation was dialysed overnight at 5° against 0·01 M-potassium phosphate buffer, pH 7·4, containing mM-ethylenediaminetetra-acetic acid (EDTA). The Amberlite resin was equilibrated with 0·01 M-potassium phosphate–1 mM-EDTA, pH 7·7, by stirring with successive portions of the buffer until fresh buffer showed no pH change. The resin was then packed under gravity at room temperature in a column 2 cm. × 6 cm. and cooled to 5°. All further operations were carried out at 5°.

The dialysed enzyme solution (30–40 ml., containing about 1 g. of protein) was cautiously adjusted to pH 7·7 with 0·1 N-NaOH (mechanical stirring) and then allowed to run through the column, slight air pressure being used if necessary to maintain a rapid flow rate (5–10 ml./min.). The column was then washed with 100 ml. of 0·01 M-potassium phosphate–1 mM-EDTA, pH 7·7, a high flow rate again being maintained. Elution of the enzyme and other proteins was accomplished with 0·1 M-potassium phosphate–1 mM-EDTA, pH 7·7, at a flow rate of about 1 ml./min. A yellow band could be observed moving down the column and all the serine-transhydroxymethylase activity was found to be associated with the yellow material, which was collected in a separate fraction of about 10 ml. volume. The specific activity of this solution varied from 348 to 760 μmoles of serine/hr./mg. of protein in various preparations. The purification achieved in this step was five- to nine-fold and the recovery about 66%. Electrophoresis of this enzyme preparation in 0·5 M-phosphate, pH 6·4, showed the presence of at least three major components. The overall purification of the enzyme achieved in the best preparation was 250-fold.

Spectrophotometric readings. A Beckman DK2 ratio recording spectrophotometer was used throughout the course of this work, the reference cell containing all the reagents present in the sample cell with the exception of the reduced pteroylglutamate. Reactions were carried out in a temperature-controlled cell at 25°.

RESULTS

DL-*Methylenetetrahydropteroylglutamic acid as substrate for serine transhydroxymethylase*

TetrahydroPGA synthesized by catalytic hydrogenation of PGA is a racemate due to the asymmetry introduced at C-6. When this DL-tetrahydroPGA reacts with formaldehyde (Fig. 1) the product is therefore also a racemate. Reaction of 0·03 mM-DL-tetrahydroPGA with mM-formaldehyde changed $\lambda_{max.}$, the wavelength of maximum absorption, from 297 to 294 mμ, with a simultaneous increase

in the maximum molecular extinction from 28 300 to 31 800 (Fig. 1*a*). The rate of this reaction is shown in Fig. 1*b*. This transformation proceeded in the same way and at the same rate whether or not serine transhydroxymethylase was present. When methylenetetrahydroPGA formation was complete, addition of glycine (final concentration 16·7 mM) in the presence of the enzyme, but not in its absence, caused a change in the spectrum. The $\lambda_{max.}$ shifted back to 296 mμ and the molecular extinction at the maximum decreased to 30 000 (Fig. 1*a*). Under the experimental conditions the latter change proceeded more rapidly than the extinction increase which occurred on combination of formaldehyde with tetrahydroPGA (Fig. 1*b*). The extinction decrease which occurred on addition of glycine indicates that DL-methylenetetrahydroPGA formed by the non-enzymic reaction of formaldehyde and DL-tetrahydroPGA is a substrate for serine transhydroxymethylase. The addition of glycine brought about only 49% reversal of the initial change, and therefore reconverted only half the methylenetetrahydroPGA into tetrahydroPGA. This incompleteness of the reaction between glycine and DL-methylenetetrahydroPGA might be due to attainment of an equilibrium in which both isomers of methylenetetrahydroPGA are reacting; but the result is also consistent with an almost quantitative reaction with one isomer.

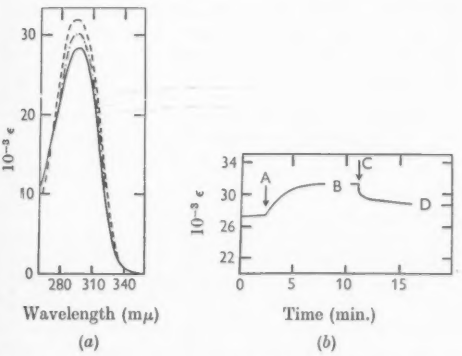

Fig. 1. DL-MethylenetetrahydroPGA as substrate for serine transhydroxymethylase. In addition to the components of the dihydroPGA-reductase system (except dihydroPGA, see Materials and Methods section) the reaction mixture contained 0·03 mM-DL-tetrahydroPGA, 0·05 M-potassium phosphate buffer, pH 7·4, and 0·48 mg. of serine transhydroxymethylase, specific activity 473 μmoles of serine/hr./mg. (*a*) —, Initial spectrum of DL-tetrahydroPGA; - - - -, spectrum (at B in *b*) of DL-methylenetetrahydroPGA formed on addition of mM-H·CHO; —·—·—, spectrum (at D in *b*) after addition of 16·7 mM-glycine. (*b*) Change in extinction at 290 mμ with time; mM-H·CHO was added at A; 16·7 mM-glycine was added at C.

DL-*Tetrahydropteroylglutamic acid as substrate for serine transhydroxymethylase*

When 0·33 mM-L-serine was added to a reaction mixture containing DL-tetrahydroPGA and serine transhydroxymethylase the spectrum changed in the same manner (Fig. 2*a*) and at about the same rate (Fig. 2*b*) as in the non-enzymic reaction of tetrahydroPGA with formaldehyde. The $\lambda_{max.}$ shifted from 297 to 295 mμ and the maximum molecular extinction increased from 28 500 to 30 600. Although this change is of the same type as produced by the action of formaldehyde (Fig. 1*a*), at equilibrium it had proceeded to only about half the extent of that change. Although this result might indicate the attainment of an equilibrium in which both isomers of tetrahydroPGA are reacting, an alternative explanation, supported by evidence given below, is that almost complete reaction occurred between serine and a single isomer of tetrahydroPGA. After this reaction had reached equilibrium, glycine (final concentration 16·7 mM) was added when the extinction decreased (Fig. 2*b*) and the first spectrum change was reversed, the molecular extinction at $\lambda_{max.}$ falling to 28 900 (Fig. 2*a*), corresponding to 80% reversal of the original change when equilibrium had been attained. The fact that 80% reversal was obtained when glycine reacted with methylenetetrahydroPGA in this experiment, compared with 49% when DL-methylenetetrahydroPGA was used in the

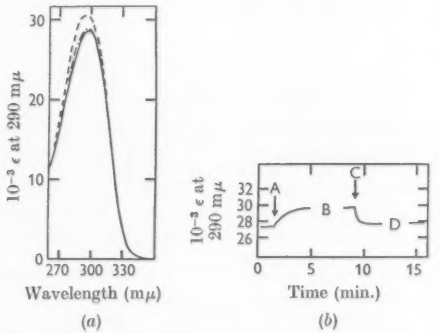

Fig. 2. DL-TetrahydroPGA as substrate for serine transhydroxymethylase. Same reaction mixture as for Fig. 1. (*a*) —, Initial spectrum of DL-tetrahydroPGA; - - - -, spectrum (at B in *b*) after addition of 0·33 mM-L-serine; —·—·—, spectrum (at D in *b*) after addition of 16·7 mM-glycine. (*b*) Change in extinction at 290 mμ with time; 0·33 mM-L-serine was added at A; 16·7 mM-glycine was added at C.

previous experiment, indicates that only one isomer could have been reacting in the previous experiment.

Active isomer of methylenetetrahydropteroylglutamic acid and of tetrahydropteroylglutamic acid

TetrahydroPGA produced by enzymic reduction of dihydroPGA differed only slightly in spectrum from the DL-tetrahydroPGA produced by catalytic hydrogenation of PGA, the enzymic product having $\lambda_{max.}$ 296–297 mμ, as compared with $\lambda_{max.}$ 298 mμ for the racemate. DihydroPGA prepared by the method of Futterman (1957) contained approx. 2·5 molecules of water when dried under the conditions used in this work, giving a molecular weight of 488·45. From this value the extinction of the enzymically formed tetrahydroPGA was found to be 28 600, in good agreement with the value of 28 400 obtained for the racemate obtained by hydrogenation over platinum catalyst.

When the isomer of tetrahydroPGA produced by enzymic reduction of dihydroPGA was treated with mM-formaldehyde the spectrum change (Fig. 3) was similar to that occurring with DL-tetrahydroPGA. The maximum molecular extinction increased from 28 500 to 32 600 and $\lambda_{max.}$ shifted from 296 to 294 mμ (Fig. 3*a*). The reaction proceeded at a similar rate (Fig. 3*b*) to that obtained with DL-tetrahydroPGA. When this reaction had been completed the addition of glycine (final concentration 16·7 mM) and serine transhydroxymethylase caused a reversal of the original spectrum change (final $\lambda_{max.}$ 296 mμ; $\epsilon_{max.}$ 29 300). No reaction occurred in the absence of the enzyme. Since the original spectrum change was reversed to the extent of 80 % as compared with 49 % for DL-methylenetetrahydroPGA, the different position of equilibrium again indicates that only one isomer of the latter compound reacted.

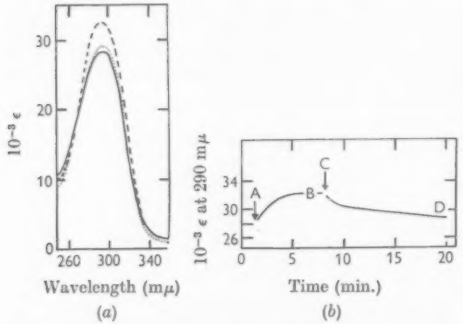

Fig. 3. The active isomer of methylenetetrahydroPGA as substrate for serine transhydroxymethylase. DihydroPGA (0·0277 mM) was reduced to tetrahydroPGA by the addition to the mixture of the components of the dihydroPGA-reductase system (see Materials and Methods section). The reaction mixture contained, in addition, 0·05 M-potassium phosphate buffer, pH 7·4, and 0·48 mg. of serine transhydroxymethylase (specific activity 473 μmoles of serine/hr./mg.). (*a*) —, Spectrum of the enzymically generated tetrahydroPGA; - - - -, spectrum (at B in *b*) of methylenetetrahydroPGA formed on addition of mM-H·CHO; . . ., spectrum (at D in *b*) after addition of 16·7 mM-glycine. (*b*) Change in extinction at 290 mμ with time; mM-H·CHO was added at A; 16·7 mM-glycine was added at C.

Spectrum changes identical with those shown in Fig. 3 occurred when the enzymically generated tetrahydroPGA reacted with serine (final concentration 0·33 mM) in the presence of serine transhydroxymethylase. The extent of the spectrum change was as great as when formaldehyde reacted with the tetrahydroPGA, confirming that only one isomer of tetrahydroPGA was present (as expected from its enzymic production) and indicating that it was completely converted into methylenetetrahydroPGA by serine transhydroxymethylase under the experimental conditions. Since this conversion of tetrahydroPGA into methylenetetrahydroPGA was twice as great as that occurring with DL-tetrahydroPGA (cf. Fig. 2), these results are also consistent with the view that only one isomer of tetrahydroPGA is active in the serine-transhydroxymethylase reaction. When methylenetetrahydroPGA formation had reached completion, addition of glycine (final concentration 16·7 mM) caused reversal of the initial spectrum change as in the experiment illustrated by Fig. 3.

Reaction of bisulphite with enzymically generated methylenetetrahydropteroylglutamic acid

The evidence so far presented that the compound obtained by the action of formaldehyde on enzymically formed tetrahydroPGA is identical with the compound obtained by the action of serine on enzymically formed tetrahydroPGA in the presence of serine transhydroxymethylase is as follows: (i) the compounds have identical spectra under the experimental conditions, and (ii) the compounds both react with glycine in the presence of serine transhydroxymethylase to yield tetrahydroPGA as indicated by spectrum changes. Further evidence to support the view that the products of the enzymic and non-enzymic reactions are identical was obtained from the action of bisulphite on the compounds.

When 0·03mM-methylenetetrahydroPGA, formed by the action of formaldehyde on tetrahydroPGA, was treated with mM-sodium bisulphite spectrophotometric measurement showed an immediate reaction, resulting in a change in the spectrum to that of tetrahydroPGA)Blakley, 1960). This was

interpreted as due to reaction of free formaldehyde with bisulphite which in turn disturbed the equilibrium shown in reaction (2) and caused the dissociation of methylenetetrahydroPGA.

The compound formed when serine reacted with tetrahydroPGA, enzymically generated from dihydroPGA, also decomposed in the presence of mM-sodium bisulphite (Fig. 4). The maximum extinction of the final product was almost identical with that of the original tetrahydroPGA, and $\lambda_{max.}$ was 293 mμ, as compared with 297 mμ for the tetrahydroPGA. This slight shift of the spectrum to shorter wavelength also occurred when mM-sodium bisulphite was added directly to tetrahydroPGA and was probably due to some slight destruction of tetrahydroPGA by the bisulphite preparation. Apart from this small discrepancy, the behaviour of the compound produced by the action of serine transhydroxymethylase was identical with that of methylenetetrahydroPGA formed by the action of formaldehyde.

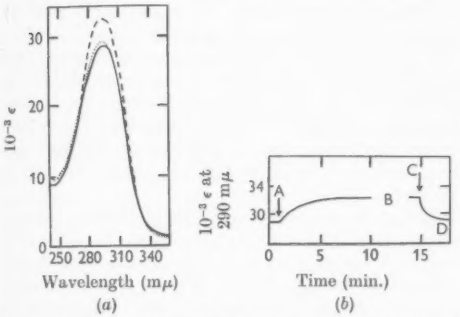

Fig. 4. Reaction of bisulphite with enzymically generated methylenetetrahydroPGA. The active isomer of tetrahydroPGA was generated in the same reaction mixture as used for Fig. 3. (*a*) ——, Spectrum of enzymically generated tetrahydroPGA; - - - -, spectrum (at B in *b*) of methylenetetrahydroPGA formed after addition of 0·33 mM-L-serine; . . ., spectrum (at D in *b*) after addition of mM-$NaHSO_3$. (*b*) Change in extinction at 290 mμ with time; 0·33 mM-L-serine was added at A; mM-$NaHSO_3$ was added at C.

D-*Serine in the transhydroxymethylase reaction*

Previous evidence has indicated that D-serine does not participate in the serine-transhydroxymethylase reaction (e.g. Blakley, 1954). Similar results were obtained in spectrophotometric experiments. Addition of D-serine to DL-tetrahydroPGA or optically active tetrahydroPGA in the presence of serine transhydroxymethylase produced no spectrum change. A mixture of D- and L-serine produced a spectrum change proceeding at the same rate as with L-serine alone. Thus it is confirmed that D-serine neither participates in nor inhibits the transhydroxymethylase reaction.

Equilibrium constant of the transhydroxymethylase reaction

The progress of the transhydroxymethylase reaction (2) can be followed spectrophotometrically, and since the molecular extinction at any particular wavelength was known for both tetrahydroPGA and methylenetetrahydroPGA the ratio of the concentrations of these compounds could be obtained from the extinction observed at equilibrium (cf. Blakley, 1959). The situation is complicated, however, by the simultaneous occurrence of the equilibrium shown in reaction (1) in the reaction mixture. Alexander & Greenberg (1956) give a value of $2{\cdot}76 \times 10^3$ for the equilibrium constant K_3 of the reaction

$$\text{Glycine} + \text{H·CHO} \rightleftharpoons \text{serine}. \qquad (3)$$

Table 1. *Equilibrium constant for the serine-transhydroxymethylase reaction*

The reaction mixture contained, in addition to the components required to generate 0·03 mM-tetrahydroPGA from dihydroPGA (see Materials and Methods section), 50 mM-potassium phosphate buffer, pH 7·4, 75 units of serine transhydroxymethylase/ml., specific activity 473 μmoles of serine/hr./mg. of protein, 1·67 mM-L-serine and glycine in the concentration indicated. The dihydroPGA-reductase reaction was allowed to proceed to completion before commencing the serine-transhydroxymethylase reaction by addition of the serine.

Serine concn. (mM)		Glycine concn. (mM)		TetrahydroPGA / MethylenetetrahydroPGA	
Initial	Equilibrium	Initial	Equilibrium		K_2
1·67	1·54	5	5·13	0·39	0·117
1·67	1·54	5	5·13	0·40	0·120
1·67	1·61	10	10·1	0·42	0·069
1·67	1·61	10	10·1	0·92	0·152
1·67	1·63	15	15	0·92	0·100
1·67	1·63	15	15	0·69	0·075
1·67	1·64	20	20	0·80	0·066
1·67	1·64	20	20	1·33	0·109
1·67	1·65	25	25	1·8	0·119
1·67	1·65	25	25	1·25	0·082
1·67	1·65	30	30	1·8	0·099
1·67	1·65	30	30	1·27	0·070

For any selected initial concentration of serine and glycine the equilibrium concentration of these compounds can be calculated with this value of K_3, provided that the concentrations of tetrahydroPGA and methylenetetrahydroPGA are low compared with those of the amino acids. Calculated equilibrium concentrations of serine and glycine corresponding to various initial concentrations are shown in Table 1.

Because of the low concentration of tetrahydroPGA reaction (2) will not displace the concentrations of serine and glycine significantly from their initial value. The difference between the initial and equilibrium values of the amino acids therefore gives some indication of the extent to which reaction (1) will displace the equilibrium of reaction (2) under the experimental conditions. The comparatively slight differences between the equilibrium and initial concentrations of amino acids in Table 1 indicates that although there will be some displacement of (2) due to the dissociation of methylenetetrahydroPGA it will be sufficiently small to permit an approximate determination of K_2, the equilibrium constant of reaction (2). This may be carried out by ignoring reaction (1) and by using the calculated equilibrium concentrations of serine and glycine and the measured ratio of

$$\frac{[\text{tetrahydroPGA}]}{[\text{methylenetetrahydroPGA}]}.$$

Table 1 shows a series of values obtained for K_2 in this way, giving a mean value of $0{\cdot}098 \pm 0{\cdot}03$.

DISCUSSION

A satisfactory demonstration that the serine-transhydroxymethylase reaction proceeds according to equation (2) would require evidence that analytically pure methylenetetrahydroPGA and glycine react in the presence of the enzyme to yield L-serine and tetrahydroPGA. Moreover, the structure of the methylenetetrahydroPGA should be conclusively demonstrated by degradation studies and chemical synthesis. Both these desiderata are difficult to fulfil because of the ease with which tetrahydroPGA and methylenetetrahydroPGA decompose in the presence of oxygen. What has been achieved is that in previous work the compound formed in solution by reaction of formaldehyde with tetrahydroPGA has been identified as N^5N^{10}-methylenetetrahydroPGA primarily on the basis of a comparison of its dissociation constant with the constants of related compounds. This compound has now been shown to react with glycine in the presence of serine transhydroxymethylase, yielding a compound with an absorption spectrum identical with that of tetrahydroPGA. Furthermore, L-serine reacts with tetrahydroPGA in the presence of serine transhydroxymethylase to yield a product with an absorption spectrum identical with that of methylenetetrahydroPGA. Spectrophotometric studies indicated that this product is decomposed to tetrahydroPGA by bisulphite in the same way as methylenetetrahydroPGA.

These data may be regarded as establishing the probability that reactions (1) and (2) are correct. However, this conclusion is always subject to the possibility that a small amount of N^5-hydroxymethyltetrahydroPGA in rapid equilibrium with N^5N^{10}-methylenetetrahydroPGA is the actual molecular species reacting on the surface of the serine transhydroxymethylase.

Further substantiation of reactions (1) and (2) may perhaps be obtained by other techniques. For example, it has been pointed out that the methylenetetrahydroPGA structure of the intermediate is implied by the reported dehydrogenation of this compound to N^5N^{10}-methinetetrahydroPGA (anhydroleucovorin) by a triphosphopyridine nucleotide-linked enzyme (Huennekens, Osborn & Whiteley, 1958).

The attempt to obtain the equilibrium constant of reaction (2) was fraught with considerable technical difficulties. The concentration of tetrahydroPGA used in these studies (approx. 0·03 mM) gave an extinction of approx. 0·86. Complete conversion into methylenetetrahydroPGA caused an increase in the extinction (to 0·96), but complete enzymic conversion was possible only when the active isomer of tetrahydroPGA was used. The change in extinction (0·86 to 0·91) obtained with DL-tetrahydroPGA was too small to enable useful measurements for calculation of K_2. The active isomer was therefore generated with dihydropteroylglutamic reductase, but it was necessary to make the assumption that this reduction was complete so that the concentration of the tetrahydroPGA formed could be assumed to be equal to the concentration of dihydroPGA added. There is good evidence that this is the case from the work of Heunnekens *et al.* (1958), and from unpublished data obtained in this laboratory.

Even with the active isomer, large errors were introduced into the value of K_2 by relatively small errors in the extinction measurements. Thus under conditions where the equilibrium concentrations of tetrahydroPGA and methylenetetrahydroPGA were equal the extinction should be 0·91. A reading of 0·90 would give a value of

$$\frac{0{\cdot}06}{0{\cdot}04} = 1{\cdot}5 \text{ for } \frac{[\text{tetrahydroPGA}]}{[\text{methylenetetrahydroPGA}]}$$

and thus an error of 50 % in the value of K_2. It is not surprising therefore that the values of K_2 showed considerable scatter, as seen in Table 1.

Apart from these technical difficulties there is also the problem that reaction (1) occurs under the experimental conditions simultaneously with reaction (2), so that the spectrophotometric measurements reflect both reactions (1) and (2). For the purpose of the determination of K_2 it was assumed that reaction (1) did not significantly disturb the equilibrium in reaction (2). The ground for this assumption was the calculation from the data of Alexander & Greenberg (1956) that the concentrations of serine and glycine were not seriously changed due to reaction (1). A mean value of 0·098 was obtained for K_2, and since K_1, the equilibrium constant for reaction (1), is $3{\cdot}2 \times 10^4$ M (Blakley, 1960), this gives a value for K_3 (the equilibrium constant for reaction 3) of $3{\cdot}1 \times 10^3$ M ($K_1 \times K_2$). This is in good agreement with the value of $2{\cdot}76 \times 10^3$ reported by Alexander & Greenberg (1956).

SUMMARY

1. Serine transhydroxymethylase has been further purified five- to nine-fold by the use of ion-exchange columns. By this additional step the overall purification of the enzyme from rabbit-liver extracts is about 250-fold. The behaviour of the enzyme on ion-exchange resins indicates that it is a basic protein with isoelectric point above pH 7·7.

2. The spectrophotometric evidence indicates that one isomer of DL-methylenetetrahydropteroylglutamic acid, produced by the action of formaldehyde on DL-tetrahydropteroylglutamic acid, is a substrate for serine transhydroxymethylase.

3. Similar evidence has been obtained which indicates that only one isomer of tetrahydropteroylglutamic acid participates in the serine-transhydroxymethylase reaction. This isomer is formed by dihydropteroylglutamic reductase from dihydropteroylglutamic acid and in the presence of serine is converted by serine transhydroxymethylase into a product with an absorption spectrum identical with that of methylenetetrahydropteroylglutamic acid. Like methylenetetrahydropteroylglutamic acid the product is instantly converted into tetrahydropteroylglutamic acid in the presence of bisulphite.

4. The equilibrium constants have been obtained for the serine-transhydroxymethylase reactions from spectrophotometric data.

I am greatly indebted to Mr J. McKeough for all preparations of serine transhydroxymethylase up to the stage preceding ion-exchange treatment, for enzyme assays and for other technical assistance.

REFERENCES

Alexander, N. & Greenberg, D. M. (1956). *J. biol. Chem.* **220**, 775.
Blakley, R. L. (1954). *Biochem. J.* **58**, 448.
Blakley, R. L. (1957). *Biochem. J.* **65**, 331.
Blakley, R. L. (1958). *Nature, Lond.*, **182**, 1719.
Blakley, R. L. (1959). *Biochem. J.* **72**, 707.
Blakley, R. L. (1960). *Biochem. J.* **74**, 71.
Futterman, S. (1957). *J. biol. Chem.* **228**, 1031.
Huennekens, F. M., Osborn, M. J. & Whiteley, H. R. (1958). *Science*, **128**, 120.
Osborn, M. J. & Huennekens, F. M. (1958). *J. biol. Chem.* **233**, 969.
Porath, J. (1957). *Ark. Kemi*, **11**, 97.

Biochem. J. (1960) **77**, 465

Purification and Properties of a Protease from the Gut of *Etroplus suratensis*

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The proteases of fishes form a group in the field of proteolytic enzymes where relatively little work has been undertaken. Generally, investigations have been carried out hitherto on the distribution of proteolytic enzymes in various fishes, particularly in their digestive tracts (Buchs, 1954; Hamoir, 1955). However, purification of these enzymes appears to have been done only to a limited extent. The main objective of these investigations has been the possible use of these enzymes as a cheap substitute for pancreatin, which is used industrially as a leather bate.

The crystallization of pepsin from the gut of salmon by Norris & Elam (1939) and the isolation of halibut, shark and tuna pepsins by Eriksen (1943), Sprissler (1942) and Norris & Mathies (1953)

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respectively, constitute a few of the isolated cases where a high degree of purification of a fish protease has been achieved. A study of trypsin-like fish proteases acting at alkaline pH therefore appeared logical. A preliminary survey conducted in this laboratory (Saroja Sundaram, 1959) showed that the gut of the freshwater fish *Etroplus suratensis* constituted a convenient source of such an enzyme. This paper embodies the results of a detailed study on the purification and properties of the *Etroplus* enzyme.

EXPERIMENTAL

Materials

Fish gut. Freshly caught fish (*Etroplus suratensis*) were obtained from the Fisheries Department, Government of Madras. After washing them well in running tap water their guts were removed and the food particles in them were gently squeezed out.

Adsorbant. The adsorbant used in this study was tricalcium phosphate gel prepared by the method of Tsuboi & Hudson (1954).

Soya-bean trypsin inhibitor. A solution of soya-bean trypsin inhibitor (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) of the required concentration was prepared in 2·5 mM-HCl.

Buffers. In most of the experiments, the buffer system was chosen from one of the following two series. Buffer series 1 consisted of three buffers (Britton, 1942): pH 2·0–6·0, 0·2N-citric acid–0·2N-sodium hydroxide; pH 6·9–11·82, 0·2N-dipotassium hydrogen phosphate–0·2N-sodium hydroxide; pH 7·46–10·03, 0·2N-boric acid–0·2N-sodium hydroxide. Buffer series 2 also comprised three buffers (Colowick & Kaplan, 1955): pH 2·2–6·0, 0·2M-potassium hydrogen phthalate–0·2M-hydrochloric acid; pH 7·2–8·4, 0·2M-tris–0·2M-hydrochloric acid; pH 8·6–10·6, 0·2M-glycine–0·2M-sodium hydroxide.

Protein substrates

Casein. Hammarsten casein was dissolved in 0·05N-sodium hydroxide to give a solution of the required concentration.

Haemoglobin. Haemoglobin (Eastman Kodak Co.) was dissolved in 0·05N-sodium hydroxide to give a solution of the required concentration.

Egg albumin. A 10% (w/v) solution of egg albumin (Merck) in 0·05N-sodium hydroxide was prepared.

Bovine-serum albumin. A 10% (w/v) solution of bovine-serum albumin (The Armour Laboratories, Chicago, Ill., U.S.A.) was prepared in 0·05N-sodium hydroxide.

Heat-denatured egg albumin and heat-denatured bovine-serum albumin. These were prepared by keeping the respective protein solutions at 100° for 5 min. and by subsequent cooling.

Synthetic substrates

The following synthetic peptides, kindly supplied by Dr E. L. Smith, Salt Lake City, Utah, U.S.A., were used: α-benzoylargininamide–HCl, benzoxycarbonylglycyl-L-phenylalaninamide and benzoxycarbonyl-L-glutamyl-L-tyrosine. L-Leucylglycylglycine and L-leucylglycine were products of A. F. Hoffman La Roche Co. Ltd., Basle, Switzerland. Solutions of these peptide substrates were prepared in boric acid (0·2N)–sodium hydroxide (0·2N) buffer, pH 9·5 (Britton, 1942).

Methods

Determination of enzyme activity. The incubation system for the estimation of proteolytic activity of the enzyme during the purification procedure consisted of 1·8 ml. of boric acid–sodium hydroxide buffer (Britton, 1942), pH 9·5, 1 ml. of 10% (w/v) casein solution and 0·2 ml. of enzyme solution. The mixture of buffer and substrate solution was first incubated at 37° for 20 min. and then the enzyme extract was added and the reaction was allowed to proceed for 30 min. at 37°. For the blank, water was added instead of the enzyme extract. At the end of incubation, the reaction was stopped by adding 1 ml. of 10% (w/v) trichloroacetic acid and, after standing for 30 min., the mixture was made up to 10 ml. with water and filtered. The extent of proteolysis during the incubation period was determined on suitable portions of the filtrate as follows: to the portion, water was added to bring the volume to 3·0 ml., followed by 4 ml. of 0·5N-NaOH and 1 ml. of the thrice-diluted phenol reagent (Folin & Ciocalteu, 1927). After thorough mixing of the solution and standing for 10 min., the blue colour formed was measured at 650 mμ in a Lumetron Colorimeter (model 400A, Photovolt Corporation, New York City). A standard curve was constructed with pure tyrosine.

The enzyme activity is expressed in units, a unit being defined as the amount of enzyme that liberates (in 1 min.) products not precipitated by trichloroacetic acid which give the same colour with phenol reagent as 1 μg. of tyrosine.

Determination of protein nitrogen in the enzyme extracts. In the initial stages, the nitrogen content of the enzyme extracts was determined by the micro-Kjeldahl method (Hawk, Oser & Summerson, 1947) and, in the later stages of the enzyme purification, where the concentration of the protein was low the nesslerization method was adopted for the estimation of protein nitrogen (Hawk *et al.* 1947).

RESULTS

Purification of the fish protease. The details of the various stages of the purification procedure and the results obtained in a typical fractionation experiment are summarized in Table 1. Nearly 200-fold purification of the enzyme could be obtained by the method adopted, with a yield of 21%. The supernatant after step 5 was used throughout for the study of the properties of the enzyme.

Optimum pH. In studies on the pH optimum of the enzyme, both buffer series 1 and 2 were employed and the results are presented in Fig. 1. The fish protease showed maximum activity between pH 9·0 and 10·5. The same results are obtained irrespective of whether buffer series 1 or 2 was employed and whether casein or haemoglobin was the substrate.

Table 1. *Purification of the protease of* Etroplus *gut*

Fresh gut (160 g.) was added to 700 ml. of chilled 2·5% NaCl solution and mixed in a Waring Blendor for 2 min. Celite (10 g.) was added and the mixture filtered under suction through a bed of Celite. The clear yellowish red filtrate was the crude extract. All procedures were carried out at 5°, and, except where otherwise stated, all precipitates were centrifuged at 13 500 *g* for 10 min.

Step no.	Details of purification	Total vol.	Total activity	Protein nitrogen (mg.)	Specificity activity (units/mg. of protein)	Yield (%)
1	Crude extract	700	280 000	1242·5	230	95
2	Supernatant after extract had been dialysed against 1% NaCl for 60 hr. and then centrifuged	705	257 325	629·7	822	88
3	Supernatant was treated with $(NH_4)_2SO_4$ to 0·8 saturation and kept 24 hr. The ppt. in 125 ml. of 2·5% NaCl was dialysed against 1% NaCl	127	125 000	21·47	5 821	42
4	Ppt. formed from (3) by 0·3–0·6 saturation with $(NH_4)_2SO_4$ was dissolved in 30 ml. of 2·5% NaCl and dialysed	31	60 000	5·5	11 270	21
5	An equal volume of calcium phosphate gel was added to solution (4). After 24 hr. the mixture was centrifuged at 1800 *g* for 10 min. and the supernatant was examined	62	60 000	1·27	48 540	21

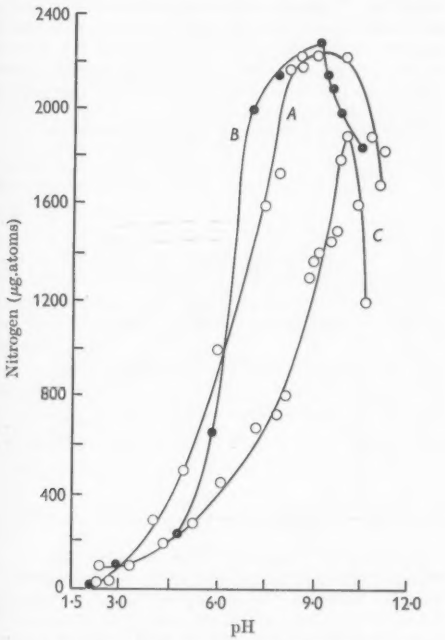

Fig. 1. Influence of hydrogen-ion concentration on the activity of *Etroplus* protease. The incubation systems comprised 2 ml. of the appropriate buffer, 1 ml. of 5% (w/v) substrate solution and 0·1 ml. of the purified enzyme. Incubation was at 37° for 30 min. Substrates: *A*, casein, buffer series 1; *B*, casein, buffer series 2; *C*, haemoglobin, buffer series 2.

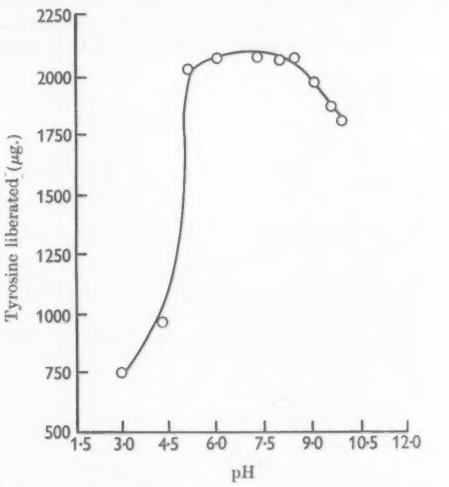

Fig. 2. Stability of *Etroplus* protease at different pH values. A portion (0·3 ml.) of the purified enzyme was incubated with 1·2 ml. of the appropriate buffer (series 2) for 60 min. at 37°. Part (0·5 ml.) of the mixture was then incubated with 1 ml. of 5% casein solution and 2 ml. of boric acid–sodium hydroxide buffer, pH 9·5.

The stability of the enzyme preparation at different pH values was studied with buffer series 1 and the result obtained is presented in Fig. 2. As can be seen from the figure, the enzyme was found to be most stable over the range pH 6·0–8·5.

Kinetics of enzymic action

Time course and order of the reaction. The time course of proteolysis with 500, 250, 125 and 62·5 mg. of casein in 15 ml. of the incubation mixture is represented in Fig. 3. With 500, 250 and 125 mg. of the substrate, the reaction appeared to follow almost zero-order kinetics over a considerable period of time (at least for the first hour), whereas when the amount of substrate is 62·5 mg. the reaction tends to obey more the requirements of a first-order reaction. At higher concentrations of the substrate, the reaction conformed to zero-order kinetics for longer periods than at the lower concentration.

Effect of enzyme concentration on reaction rate. The effect of enzyme concentration on the rate of proteolysis was determined by varying the enzyme concentration from 0 to 0·0058 mg. of protein nitrogen/2·5 ml. The enzyme concentration–reaction velocity plot exhibited linearity over the range of concentration studied.

Michaelis constant. The dependence of enzymic activity upon substrate concentration was studied at two enzyme concentrations, and from the Lineweaver & Burk plot (Fig. 4) the Michaelis constant was evaluated, the mean value being 22·5 mg.atoms of casein nitrogen/l. The Michaelis constants for proteases acting on proteins have not been evaluated with much accuracy, although in recent years this constant has been studied in detail with

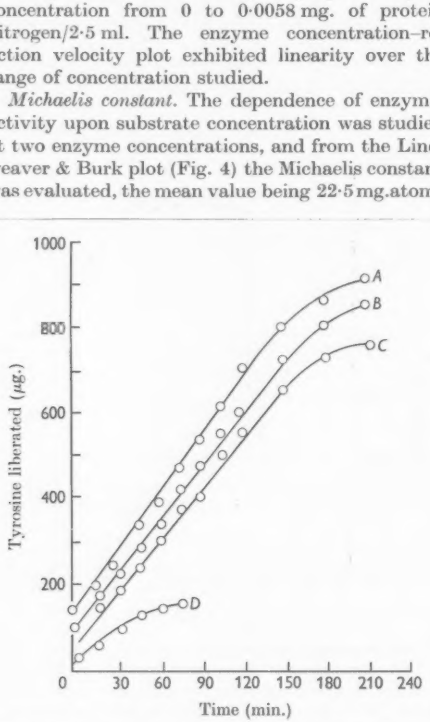

Fig. 3. Time course of casein hydrolysis by *Etroplus* protease. The incubation mixture comprised 5 ml. of casein solution of the appropriate concentration, 9 ml. of boric acid–sodium hydroxide buffer, pH 9·5, and 1 ml. of the enzyme (purified enzyme diluted eight times with buffer). Incubation was at 37° and at different intervals 1 ml. portions of the reaction mixture were withdrawn. Substrate concentration (mg. of casein/15 ml. of the incubation mixture): *A*, 500; *B*, 250; *C*, 125; *D*, 62·5.

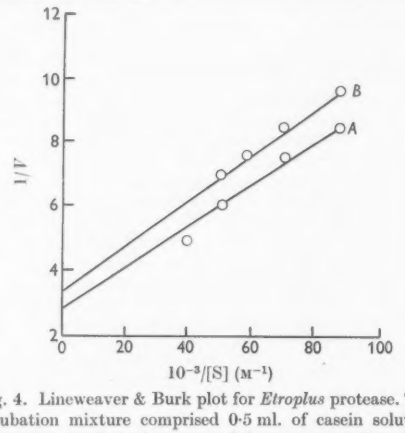

Fig. 4. Lineweaver & Burk plot for *Etroplus* protease. The incubation mixture comprised 0·5 ml. of casein solution containing the desired amount of the protease in 2·5 ml. of boric acid–sodium hydroxide buffer, pH 9·5, and 0·2 ml. of enzyme. Incubation was for 15 min. at 37°. *A*, Purified enzyme diluted four times; *B*, purified enzyme diluted eight times. [S], Concentration of substrate expressed as mg.atoms of casein nitrogen/l. (mM). *V*, Initial velocity of reaction expressed as tyrosine (μmoles) liberated/min.

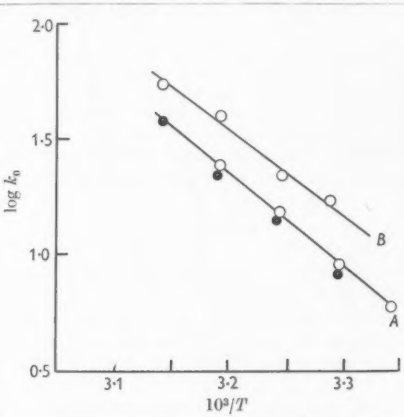

Fig. 5. Influence of temperature on the velocity constant of *Etroplus* protease. The incubation mixture comprised 0·5 ml. of 10% (w/v) casein solution, 2·3 ml. of boric acid–sodium hydroxide buffer, pH 9·5, and 0·2 ml. of enzyme. Incubation was for 15 min. at the desired temperature. *A*, Purified enzyme diluted four times; *B*, purified enzyme twice diluted. ○ and ● in curve *A* represent values obtained from two separate but identical experiments. *T*, Temperature of incubation. k_0, Zero-order velocity constant (μg. of tyrosine liberated/min.).

synthetic substrates (Hofmann & Bergmann, 1941; Kaufman, Neurath & Schwert, 1949; Kaufman, Neurath, Schwert & Snoke, 1948; MacAllister, Harmon & Niemann, 1949).

Influence of temperature on reaction rate and energy of activation. The proteolysis of casein by the purified enzyme was studied at 25°, 30°, 35°, 40°, 45° and 50° and the results are presented in Fig. 5. The experimental conditions in this case were so chosen that the reaction followed zero-order kinetics and the period of incubation was as short as possible to prevent enzyme inactivation. The straight-line nature of the plot of $1/T$ against $\log k_0$ (Fig. 5) satisfies the requirements of the Arrhenius equation.

The close agreement of the several values calculated from the Arrhenius equation for activation energy is also indicative of the validity of the results obtained. The mean value of the activation energy for *Etroplus* protease was found to be 18 149 cal./mole.

Substrate specificity of the enzyme. The proteolytic activity of the enzyme on various proteins (casein, haemoglobin, egg albumin, bovine-serum albumin, heat-denatured egg albumin and heat-denatured bovine-serum albumin) was estimated during a 2 hr. incubation period. The results are presented in Fig. 6. Casein was the protein most susceptible to the protease; among the other substrates, egg albumin and bovine-serum albumin were not attacked even after 2 hr. of incubation. However, heat-denaturation of these proteins before incubation made them subsequently susceptible to the enzyme.

The results presented in Table 2 show that the enzyme attacked α-benzoyl-L-argininamide, which is a specific substrate for trypsin, whereas benzoxycarbonylglycyl-L-phenylalaninamide, which is a typical chymotrypsin substrate, was completely resistant.

Further experiments at pH 9·5 showed that benzoxycarbonyl-L-glutamyl-L-tyrosine and L-leucylglycine, which are substrates for pepsin and peptidase respectively, were not hydrolysed. The paper-chromatographic method of Giri (Block, Durrum & Zweig, 1958) was used to test for hydrolysis products.

Effect of activators and inhibitors on the activity of the fish protease. The effect of various metal ions and other miscellaneous substances on the proteolytic activity of the fish protease was studied by incubating the test compounds at the required concentration with the enzyme for 15 min. before the addition of the substrate (casein).

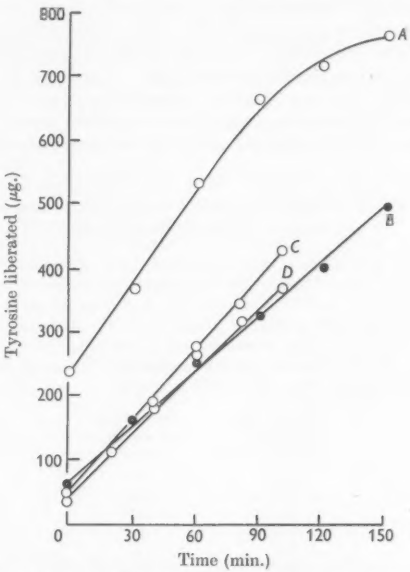

Fig. 6. Action of *Etroplus* protease on protein substrates. The incubation mixture comprised 3 ml. of 10% (w/v) protein solution, 5·8 ml. of boric acid–sodium hydroxide buffer, pH 9·5, and 0·2 ml. of purified enzyme. Incubation was at 37°. Portions (1 ml.) were withdrawn at different time intervals. *A*, Casein; *B*, haemoglobin; *C*, heat-denatured bovine-serum albumin; *D*, heat-denatured egg albumin.

Table 2. *Action of* Etroplus *protease on α-benzoyl-L-argininamide and benzoxycarbonylglycyl-L-phenylalaninamide*

The incubation system comprised 1·8 ml. of boric acid–NaOH buffer, pH 9·5, containing the substrate and 0·2 ml. of enzyme. Incubation was at 37° for the time indicated, at the end of which 1 ml. of 10% trichloroacetic acid was added and the ammonia estimated by Folin's aeration method modified by Van Slyke & Cullen (1914).

Period of incubation (min.)	Substrate	NH_3 liberated (as μg.atoms of nitrogen)	Substrate split (%)
30	α-Benzoyl-L-argininamide (8 μmoles)	2·05	25·6
60		3·97	49·6
30	Benzoxycarbonylglycyl-L-phenylalaninamide (10 μmoles)	0·07	Negligible
60		0·01	Negligible

The results obtained for the effect of various metal ions on the activity of protease are presented in Table 3. The enzyme was unaffected by the metal ions tested even at the relatively high concentration employed (0·01 M), except for Mn^{2+} ions, which stimulated the activity, and Fe^{3+} and Zn^{2+} ions, which slightly inhibited the enzyme.

Among the other miscellaneous activators and inhibitors tested, sulphanilamide, *p*-aminobenzoic acid, urea and potassium thiocyanate, at 0·01 M concentration, exerted little or no effect on the enzyme. *O*-Iodosobenzoate and *p*-chloromercuribenzoate at a concentration of mM were virtually without any effect. This, as well as the fact that none of the reducing agents and sulphydryl compounds had any marked stimulating effect and that the oxidizing agent ferricyanide, even at 0·01 M concentration, lowered the activity by only 14 %, shows that the protease is not a sulphydryl enzyme. However, glutathione at 0·01 M concentration activated the enzyme by about 30 %. Both dipyridyl and ethylenediaminetetra-acetate had no effect on the enzyme. The inference is warranted therefore that no metal activator is essential for the *Etroplus* protease.

The results of studies with the natural soya-bean trypsin inhibitor presented in Table 4 show that at a concentration of 20 μg. of the inhibitor/3 ml., the *Etroplus* enzyme was inhibited to the extent of 84 %.

Table 3. *Influence of metal ions on the protease of the gut of* Etroplus suratensis

The incubation system comprised 2 ml. of boric acid–NaOH buffer, pH 9·5, 0·3 ml. containing the test substance (0·1 M), 0·5 ml. of 10 % casein solution (0·5 ml. of water in the blank) and 0·2 ml. of purified enzyme. The volume (0·3 ml.) of the test substance was replaced by 0·3 ml. of water in the control. Incubation was for 60 min. at 37°. The proteolysis was determined as usual.

Metal ion (at 0·01 M concentration)	Percentage of control activity
Hg^{2+}	96
Mg^{2+}	100
Mn^{2+}	140
Co^{2+}	97
Cu^{2+}	107
Fe^{3+}	86
Zn^{2+}	90
Ca^{2+}	97

Table 4. *Effect of soya-bean trypsin inhibitor on* Etroplus *protease*

The incubation system comprised 2·3 ml. of boric acid–NaOH buffer, pH 9·5, containing the desired amount of trypsin inhibitor, 0·5 ml. of 10 % casein solution and 0·2 ml. of purified enzyme (0·2 ml. of water in the blank). Incubation was for 30 min. at 37°. The extent of proteolysis was estimated as usual in the trichloroacetic acid filtrate.

Concn. of inhibitor (μg./3 ml.)	Percentage inhibition
2	32
4	46
6	71
8	71
10	76
15	79
20	84
30	84

DISCUSSION

The *Etroplus* protease differs from mammalian trypsin in having a slightly higher pH optimum. However, several instances are known of fish proteases having pH optima around 9 (Labarre, Tremblay & Dussalt, 1951; Akiya, Teshima & Tshikawa, 1949; Utjino & Nishiwaki, 1951), and a proteolytic enzyme exhibiting maximum activity at as high as pH 12·5 has been reported.

The susceptibility of both egg albumin and bovine-serum albumin to the enzyme only after heat-denaturation recalls a parallel observation made in respect of trypsin (Sri Ram & Maurer, 1957) and is indicative of the similarity between the two enzymes. This is further supported by the fact that the *Etroplus* protease attacks only the trypsin substrate. Activation and inhibition studies show that it does not require a metal ion for its activity and is not a sulphydryl enzyme. The inhibition by soya-bean trypsin inhibitor corroborates the conclusion that the enzyme is a protease of the trypsin type.

SUMMARY

1. From the gut of *Etroplus suratensis* a protease acting at alkaline pH has been purified 207-fold with a yield of about 20 %.

2. The enzyme exhibits maximum activity at pH 9·5–10·5 and is most stable at pH 6·0–8·5. The time course of the proteolysis of casein by the enzyme and the enzyme concentration–reaction velocity relationship have also been investigated.

3. The Michaelis constant for the protease evaluated from the Lineweaver & Burk plot is 22·5 mg.atoms of casein nitrogen/l.

4. The relationship between the temperature and velocity reaction follows the requirements of the Arrhenius equation over the range 25–50°. The value for the apparent energy of activation calculated from these data is 18 149 cal./mole.

5. Casein is the most susceptible protein substrate; haemoglobin is less susceptible and bovine-serum albumin and egg albumin are attacked only after heat-denaturation.

6. The protease attacks only α-benzoyl-L-argininamide among the several peptides tested, which shows that it is of the trypsin type.

7. The enzyme is practically unaffected by any of a variety of compounds tested. It is not a sulphydryl enzyme and does not require metal ions for activity and is inhibited by soya-bean trypsin inhibitor.

The generosity of the Fisheries Department, Government of Madras, in supplying the fish material used in this investigation is gratefully acknowledged. Also, we are deeply indebted to Professor E. L. Smith, College of Medicine, Salt Lake City, Utah, U.S.A., for the gift samples of the synthetic protease substrates used in this study and to Dr E. Raghupathy for going through the manuscript. The authors wish to thank the University of Madras for the award of a research studentship to one of them (S.S.) and for kind permission to publish the results, which form part of a thesis approved for the degree of Master of Science.

REFERENCES

Akiya, S., Teshima, S. & Tshikawa, Y. (1949). *Symp. enzyme Chem. Japan*, **3**, 43.
Block, R. J., Durrum, E. L. & Zweig, G. (1958). *A Manual of Paper Chromatography and Paper Electrophoresis*. New York: Academic Press Inc.
Britton, H. T. S. (1942). *Hydrogen Ions*, vol. 1, p. 300. London: Chapman and Hall Ltd.
Buchs, S. (1954). *Z. vergl. Physiol.* **36**, 165.
Colowick, S. P. & Kaplan, N. O. (1955). *Methods in Enzymology*, vol. 1, p. 138. New York: Academic Press Inc.
Eriksen, N. (1943). Thesis: University of Washington.
Folin, O. & Ciocalteu, V. (1927). *J. biol. Chem.* **73**, 627.
Hamoir, G. (1955). *Advanc. Protein Chem.* **10**, 227.
Hawk, P. B., Oser, B. L. & Summerson, W. H. (1947). *Practical Physiological Chemistry*. New York: Blackiston Co.
Hofmann, K. & Bergmann, M. (1941). *J. biol. Chem.* **138**, 231, 243.
Kaufman, S., Neurath, H. & Schwert, G. W. (1949). *J. biol. Chem.* **177**, 793.
Kaufman, S., Neurath, H., Schwert, G. W. & Snoke, J. E. (1948). *J. biol. Chem.* **172**, 221.
Labarre, J., Tremblay, J. L. & Dussalt, H. P. (1951). *Rev. Canad. Biol.* **10**, 140.
MacAllister, R. V., Harmon, K. M. & Niemann, C. (1949). *J. biol. Chem.* **177**, 767.
Norris, E. R. & Elam, D. W. (1939). *Science*, **90**, 399.
Norris, E. R. & Mathies, J. C. (1953). *J. biol. Chem.* **204**, 673.
Saroja Sundaram (1959). *J. sci. industr. Res.* **18C**, 182.
Sprissler, G. P. (1942). Dissertation: Catholic University of America, Washington, D.C.
Sri Ram, J. & Maurer, P. H. (1957). *Arch. Biochem. Biophys.* **70**, 185.
Tsuboi, K. K. & Hudson, P. B. (1954). *Arch. Biochem. Biophys.* **53**, 341.
Utjino, S. & Nishiwaki, I. (1951). *Acta Sch. med. Univ. Kioto*, **29**, 1.
Van Slyke, D. D. & Cullen, G. E. (1914). *J. biol. Chem.* **19**, 211.

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A Protein from Leaves Catalysing the Reduction of Metmyoglobin and Triphosphopyridine Nucleotide by Illuminated Chloroplasts

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In his early experiments with isolated chloroplasts Hill (1939) was able to show that when these are illuminated in the presence of an extract of acetone-dried leaf, oxygen is evolved. The extracts therefore contained soluble material in substrate amounts capable of accepting hydrogen in the photochemical system. Although the pathway of hydrogen transport in this reaction has not yet been determined, two leaf-protein fractions have since been shown to be active catalysts of hydrogen transport from illuminated chloroplasts.

One of these, occurring in acetone-dried leaf powders, was first observed to catalyse the reduction of metmyoglobin (Davenport, Hill & Whatley, 1952). The active protein has since been obtained from pea leaves in a highly purified state, homogeneous both electrophoretically and in the ultracentrifuge (Davenport & Hill, 1960) and was shown also to promote the reduction of other haem–protein compounds including cytochrome *c* and cytochrome b_3.

Meanwhile San Pietro & Lang (1958) isolated a spinach-leaf-protein fraction active in catalysing the photochemical reduction of triphosphopyridine nucleotide. This also has since been purified (San Pietro, 1958) and named 'photosynthetic pyridine nucleotide reductase'.

During some experiments aimed at confirming the observations of San Pietro & Lang (1958) it was noticed that the capacity of leaf-protein fractions to

promote the reduction of triphosphopyridine nucleotide was always accompanied by activity towards metmyoglobin. Attempts to separate the two activities have been unsuccessful. Similarly, the 'haem–protein-reducing factor' in spite of the very different method of preparation, is active towards triphosphopyridine nucleotide. In this material also, attempts to separate the two activities have been unsuccessful. The object of the present paper is to compare metmyoglobin and triphosphopyridine nucleotide as hydrogen acceptors in the photochemical reductions, catalysed by leaf-protein fractions prepared by a variety of methods. This work has been briefly reported (Davenport, 1959*b*).

EXPERIMENTAL

Plant materials. Seedlings of *Pisum sativum*, *Spinacea oleracea* and *Beta vulgaris* (spinach beet) were raised in a cool greenhouse from seed sown in sand and irrigated with a complete nutrient solution (Hewitt, 1952). Leaves intended for protein preparations were either used immediately after harvesting or stored in sealed boxes at $-20°$ until required.

Chloroplast preparations. Chloroplasts were prepared from fresh leaves of *S. oleracea*. They were ground in a chilled mortar with about 5 times their weight of an ice-cold solution containing 0·4 M-sucrose, 0·01 M-NaCl and 0·05 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·5. After filtration through a layer of muslin, the filtrate was centrifuged at 500*g* and 0° for 2 min. and the pellet discarded. The supernatant fluid was centrifuged at 2000*g* for 10 min. to give a pellet consisting largely of whole chloroplasts. This was resuspended in the cold sucrose solution. Where broken chloroplasts (grana) were required the supernatant fluid was again centrifuged at 18 000*g* for 20 min. and the pellet resuspended as before. The suspensions of grana and chloroplasts were adjusted to a chlorophyll (a + b) concentration of about 0·6 mg./ml.

Chlorophyll concentration. This was determined in acetone extracts by the method of Mackinney (1941).

Metmyoglobin. Crystalline, sperm-whale metmyoglobin, which had been stored in 3 M-$(NH_4)_2SO_4$ solution at 0° was prepared for use and the haematin concentration estimated as described by Davenport & Hill (1960).

Other materials. Triphosphopyridine nucleotide (TPN), diphosphopyridine nucleotide (DPN), adenosine triphosphate (ATP) and adenosine 5′-phosphate (AMP) were products of Sigma Chemical Co., St Louis, Mo., U.S.A. Adenosine diphosphate (ADP) was obtained from this source and also from Boehringer und Söhne, Mannheim, Germany.

Protein determination. Protein contents of leaf preparations were estimated by the Folin-phenol-reagent method of Lowry, Rosebrough, Farr & Randall (1951). This method was calibrated against the Kjeldahl procedure of Chibnall, Rees & Williams (1943).

Photochemical-activity measurements. The photochemical reactions were carried out aerobically in silica cells of 1 cm. light path and 3 ml. capacity. Reduction of TPN or of metmyoglobin was followed in an Optica CF4 spectrophotometer as an increase in extinction at 340 mμ or 582 mμ respectively. The blank cell usually contained all the ingredients of the reaction mixture except the catalytically active leaf protein. Reaction and blank cells were illuminated by a 500 w projector lamp at 36 cm. The incident light was filtered through heat-absorbing glass (Chance ON 20) and a 1 cm. layer of 5% (w/v) cupric sulphate and the cells were immersed during illumination in a glass-sided water bath at 17°. The time course of a reduction was obtained by alternating periods of illumination with measurements of extinction.

With TPN, $\epsilon_M^{340\,m\mu}$ was taken as $6{\cdot}25 \times 10^3$ (Colowick, Kaplan & Ciotti, 1951). Under the experimental conditions described an increase in extinction of 0·1 is equivalent to the reduction of 0·048 μ-mole of TPN, independent of pH.

The increase in extinction at 582 mμ, occurring during metmyoglobin reduction, is the result of the rapid oxygenation of myoglobin produced in the reaction to oxymyoglobin (α-band maximum at 582 mμ). Since the extinction of metmyoglobin at this wavelength varies with pH (Bowen, 1949) and that of oxymyoglobin does not, the relationship between the observed increase in extinction and μmole of metmyoglobin reduced will also vary with pH. The relationship was therefore determined in each experiment by allowing the reaction to run to completion and relating the overall increase in extinction to the initial concentration of metmyoglobin determined spectrophotometrically as cyanmetmyoglobin (Davenport & Hill, 1960). The validity of this procedure depends on the absence of back reactions leading to the reoxidation of oxymyoglobin and these were never observed. Under the experimental conditions and at pH 7·7, the pH most commonly used, an increase in extinction of 0·1 was found to be equivalent to the reduction of 0·0255 μmole of metmyoglobin. This is in good agreement with the value calculated from the spectrophotometric data of Bowen (1949) for horse-heart myoglobin.

Specific activity of the leaf protein. San Pietro & Lang (1958) have defined one unit of enzyme as the amount which produces an increase in extinction of TPN of 1·0 in 10 min. at 340 mμ when the reaction mixture contains 100 μg. of chlorophyll in 3 ml. This definition has been adopted in the present work in the absence of a workable method of compensating for the variability observed in the photochemical activity of different chloroplast preparations.

RESULTS

Leaf-protein preparations

Type I. The preparation of the haem–protein reducing factor from pea leaves by ammonium sulphate fractionation of the expressed leaf juice followed by electrophoresis on paper has been described in detail (Davenport & Hill, 1960). The procedure has now also been applied, without modification, to leaves of spinach and spinach beet. Preparations made in this way will be referred to as type I.

Type II. For purposes of comparison the method used by San Pietro & Lang (1958) for the preparation of 'photosynthetic pyridine nucleotide reductase' has also been followed with leaves of pea, spinach and spinach beet. In this procedure a protein fraction precipitated from a leaf juice by cold acetone is redissolved, dialysed against dilute tris buffer, pH 8, and treated with protamine

sulphate. Active material is removed from the insoluble protamine complex by exhaustive extraction with more concentrated tris buffer. Preparations made in this way will be referred to as type II.

A number of combinations of these two preparative methods have also been examined. When type II preparations were subjected to electrophoresis on paper the material active towards metmyoglobin and TPN migrated in the manner described for preparations obtained by ammonium sulphate fractionation (Davenport & Hill, 1960). Similarly, it was found possible to effect a purification of type I preparations before electrophoresis, when these were subjected to the protamine sulphate treatment. When attempts were made to substitute ammonium sulphate fractionation for the protamine treatment with preparations from pea leaves started according to the method of San Pietro & Lang, activity towards TPN was found to be confined to the fraction precipitated above 70% saturation with respect to the salt. The activity in this fraction was rapidly lost when freshly harvested leaves were used as the starting material. By holding the leaves at $-20°$ for at least 2 days this loss of activity was found to be greatly minimized and further fractionation was then possible. This observation is exactly similar to that reported in connexion with the metmyoglobin-reducing activity (Davenport & Hill, 1960) and provided the first indication that the two activities might be properties of a single protein fraction. Modifications of the preparative methods will be referred to at the appropriate point in the text.

Reduction of triphosphopyrine nucleotide

Preparations of type I at all stages of purification were active in catalysing the reduction of TPN by illuminated chloroplasts but all were completely inactive towards DPN. The effect of increasing concentration of added leaf protein, before and after electrophoresis, upon the rate of this reaction is shown in Fig. 1 where the final preparative step gave a ninefold increase in specific activity to the high concentration of 83 units/mg. of protein. With both the crude and purified protein the reduction rate was linearly related to the concentration of added protein until the particulate photochemical system became saturated at a maximum reduction rate, in this experiment, of 184 μmoles of TPN/mg. of chlorophyll/hr. Half-maximal reduction rate was here obtained by the addition of 40 μg. of the purified protein. This, on the basis of a calculated molecular weight of 19 000 (Davenport & Hill, 1960), is equivalent to 2 μm-moles of the protein.

The highest reduction rate for TPN so far observed is 270 μmoles/mg. of chlorophyll/hr.

Effect of a phosphate acceptor system. In the experiment shown in Fig. 1 the reaction mixture was supplemented by ADP, orthophosphate and magnesium chloride since it had been found that all these are necessary for maximal activity. Arnon, Whatley & Allen (1958) have shown that the photochemical reduction of TPN catalysed by a chloroplast extract can be coupled to phosphorylation by including the above phosphate-accepting system in the reaction mixture. Inorganic phosphate was then shown to be incorporated into ATP. There have been no reports that this phosphorylation leads to an increase in the reduction rate. By contrast Arnon *et al.* (1958) found ferricyanide to be reduced more rapidly by illuminated chloroplasts when this reaction, which does not need a catalyst, is coupled to phosphorylation, and this observation has been confirmed and extended by Avron, Krogmann & Jagendorf (1958).

In the present work a stimulation of TPN reduction in the presence of the phosphate-accepting system could always be obtained with preparations of type I or II. The effect of adding the ingredients of this system either separately or in all possible combinations is shown in Table 1. Although ADP alone, or in combination with either orthophosphate or Mg^{2+} ions, gave marked stimulation, the maximum rate could only be achieved when all three were present. The table also shows that arsenate is as effective as phosphate in stimulating the reduction.

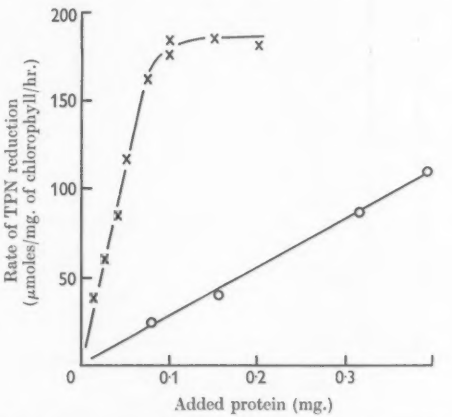

Fig. 1. Rate curves for the reduction of TPN by spinach chloroplasts catalysed by pea-leaf protein (type I). ○, Before electrophoresis; ×, after electrophoresis. Reaction mixtures (3 ml.) contained added protein as indicated, chlorophyll, 0·03 mg. and the following (in μmoles): TPN, 0·4; ADP, 0·5; $MgCl_2$, 20; Na_2HPO_4, 10; tris–HCl buffer, pH 7·7, 150; NaCl, 40. Leaf protein was omitted from the blank cell.

In the experiment shown in Table 1 the photochemical system was saturated with respect to added protein. It is in this saturation range that the stimulating effect of the phosphate-accepting system was found to be most marked. This is illustrated by the experiment shown in Fig. 2 in which a threefold stimulation of the reduction rate occurred in the presence of 0·3 mg. of protein, twofold with 0·05 mg. and no stimulation with 0·02 mg.

Of the adenosine compounds tested in the presence of orthophosphate and Mg^{2+} ions only ADP was found to be effective alone. Neither AMP nor ATP could be substituted for ADP in producing an increase in the reduction rate. When, however, AMP was supplemented by a catalytic amount of either ADP or ATP, marked stimulation occurred although the magnitude of the effect was not as great as with ADP alone (Fig. 3). This observation agrees with the finding (Mazelis, 1956) that spinach chloroplasts contain an active adenylate kinase.

Table 1. *Effect of ingredients of a phosphate-accepting system on the rate of reduction of triphosphopyridine nucleotide*

Each reaction mixture contained, in a total volume of 3 ml., spinach chloroplasts (chlorophyll content 0·029 mg.), pea-leaf protein (type I) 0·13 mg. and (in μmoles) TPN, 0·4; NaCl, 40; tris–HCl buffer, pH 8·0, 150. Where indicated the following additions were made (μmoles): ADP, 0·5; $MgCl_2$, 15; Na_2HPO_4, 8; Na_2HAsO_4, 8. The blank cell contained all the above ingredients except leaf protein and arsenate.

Additions	TPN reduced (μmoles/mg. of chlorophyll/hr.)	Percentage of control rate
None	74	100
Mg^{2+}	84	114
PO_4^{3-}	76	103
ADP	120	162
$Mg^{2+} + PO_4^{3-}$	90	120
$ADP + PO_4^{3-}$	130	176
$ADP + Mg^{2+}$	130	176
$ADP + PO_4^{3-} + Mg^{2+}$	191	259
$ADP + AsO_4^{3-} + Mg^{2+}$	195	264

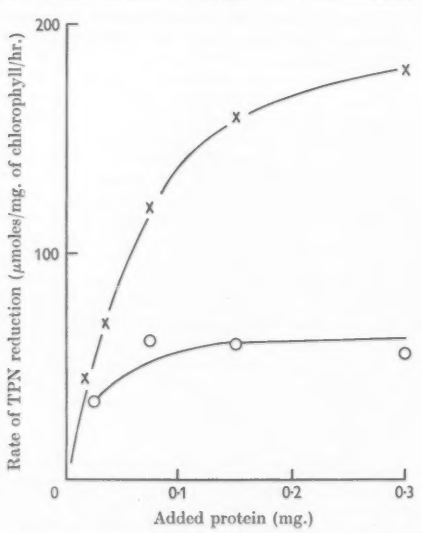

Fig. 2. Effect of a phosphate-acceptor system on TPN reduction catalysed by pea-leaf protein (type II). All reaction mixtures contained (in 3 ml.) leaf protein as indicated, spinach chloroplasts (chlorophyll content 0·016 mg.) and (in μmoles) NaCl, 40; TPN, 0·4; tris–HCl buffer, pH 7·0, 150. ×, Reaction mixtures contained in addition, ADP, 0·5; Na_2HPO_4, 15; and $MgCl_2$, 20. The blank cell contained all ingredients except leaf protein. Illumination time, 2 min.

Reduction of metmyoglobin

The activity of preparations of type I towards metmyoglobin and other haem–protein compounds as hydrogen acceptors has already been described in detail (Davenport & Hill, 1960). In the present work this activity has been compared directly with the activity towards TPN with leaf-protein preparations of both types. One comparative study of this kind, with a type II preparation from pea leaves further purified by electrophoresis on paper (Davenport & Hill, 1960), is shown in Fig. 4. Here the

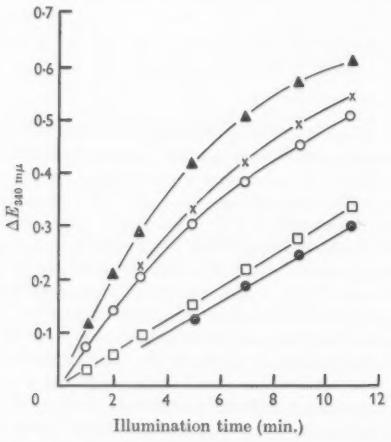

Fig. 3. Effect of adenosine derivatives on the rate of TPN reduction catalysed by pea-leaf protein (type I). All reaction mixtures contained (in 3 ml.) 0·08 mg. of leaf protein, spinach chloroplasts (chlorophyll 0·02 mg.) and (in μmoles) TPN, 0·4; tris–HCl buffer, pH 7·0, 150; NaCl, 40; Na_2HPO_4, 10; $MgCl_2$, 15, with the following additions (μmoles) ●, ATP, 0·5; □, AMP, 0·5; ○, AMP, 0·5, ATP, 0·05; ×, AMP, 0·5, ADP, 0·05; ▲, ADP, 0·5. Leaf protein and the additional ingredients were omitted from the blank cell.

effect of increasing amounts of the leaf protein on the rate of reduction of the two hydrogen acceptors is shown. At low concentrations of the leaf protein the reaction rates, expressed as μmoles of TPN or metmyoglobin reduced in unit time, are equal. Two hydrogen equivalents, however, are required in TPN reduction whereas metmyoglobin requires only one. When the reaction rates are limited by low leaf-protein concentration, therefore, TPN was twice as effective as metmyoglobin as a hydrogen acceptor in the chloroplast reaction. This relationship was found with all the active protein preparations used in making this comparison. When the reaction rates were increased by addition of more leaf protein and the photochemical system became saturated, TPN was then 1·3 times as effective (Fig. 4). This ratio varied in different experiments between 1·2 and 1·7 and this variability appeared to be determined, at least in part, by the chloroplasts used in the reaction. Thus a pea-leaf preparation of type I, assayed with spinach chloroplasts, gave a ratio of 1·3, but this was increased to 1·65 when spinach grana were substituted.

Effect of a phosphate-acceptor system. When metmyoglobin served as hydrogen acceptor in assay systems containing protein preparations of type I or II from all the plant species examined, the inclusion of ADP, orthophosphate and Mg^{2+} ions had no significant effect upon the reduction rate (Fig. 5).

Effect of TPN. With purified preparations of types I and II the rate of metmyoglobin reduction was found to be inhibited by the addition of TPN to the reaction mixture (Fig. 5). No inhibition was observed with DPN. Inhibition was further increased when the system containing metmyoglobin and TPN was supplemented by ADP, orthophosphate and Mg^{2+} ions. This result suggested that TPN was acting as a competitive inhibitor of metmyoglobin reduction. In similar experiments with crude-pea or spinach-leaf preparations (type II before protamine sulphate treatment) the opposite result was obtained, and here the addition of TPN was found to enhance the rate of metmyoglobin reduction. These divergent results were reconciled when it was found that the crude leaf extract contained a thermolabile system capable of reducing metmyoglobin when reduced TPN served as hydrogen donor. If, in the photochemical reaction, reduced TPN was produced concomitantly with the reduction of metmyoglobin, this reduced TPN could, in the presence of the 'metmyoglobin reductase', reduce a further amount of metmyoglobin to give an apparent stimulation of the rate of metmyoglobin

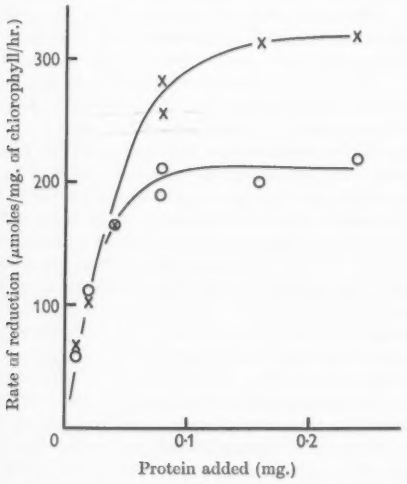

Fig. 4. Comparison of the activities of a pea-leaf-protein preparation (type II after electrophoresis) in catalysing the reduction of TPN and metmyoglobin. Reaction cell contained pea-leaf protein as indicated, spinach chloroplasts (chlorophyll, 0·015 mg.) and (in μmoles) phosphate buffer, pH 7·7 (K_2HPO_4, NaH_2PO_4); NaCl, 40 and the following: ○, TPN, 0·4; $MgCl_2$, 15; ADP, 0·5; ×, metmyoglobin, 0·26. Leaf protein was omitted from the blank cells.

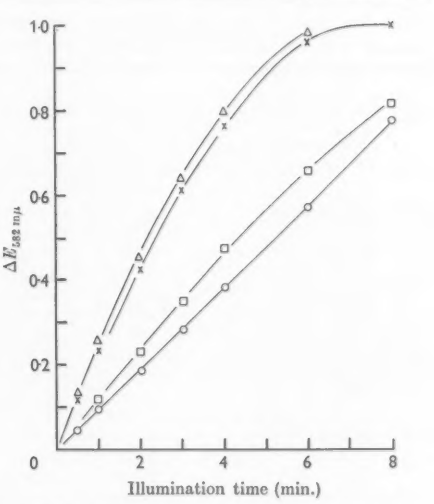

Fig. 5. Effect of TPN upon the time course of metmyoglobin reduction. Reaction mixtures contained (in 3 ml.) pea-leaf protein (type I) 0·12 mg.; spinach chloroplasts (chlorophyll, 0·031 mg.) and (in μmoles) metmyoglobin, 0·255; phosphate buffer, pH 7·7 (K_2HPO_4, Na_2HPO_4); NaCl, 40; $MgCl_2$, 20; with the following additions: ×, none; △, ADP, 0·5; □, TPN, 0·4; ○, TPN, 0·4, ADP, 0·5. The blank cell contained neither leaf protein nor the additional ingredients.

reduction. In support of this interpretation was the observation that when the photochemical reduction in this system was stopped by turning off the light the increase in extinction at 582 mμ continued for a time in darkness. An attempt was made to use this 'metmyoglobin reductase' to measure the rate of reduction of TPN in the presence of metmyoglobin but it was found that the crude leaf extracts could also oxidize reduced TPN by pathways other than that where metmyoglobin was the hydrogen acceptor (Davenport, 1959*a*). The capacity to oxidize reduced TPN was found to be absent from the purified preparations of type I or II.

Absorption spectra

The absorption spectra of preparations of type I have already been described (Davenport & Hill, 1960). The reddish-brown colour of the protein solution is the result of increased absorption in the blue region of the spectrum together with the occurrence of two diffuse absorption bands at 465 and 422 mμ. In Fig. 6 this spectrum of a preparation from pea leaves is compared with that of a type II preparation, also from pea leaves, which had been further purified by electrophoresis on paper. This was the preparation used in obtaining the data for Fig. 4. In order to facilitate comparison the curves shown in Fig. 6 are 'characteristic absorption spectra' with log extinctions as ordinates. Plotted in this way the shape of the curve is characteristic of the absorbing substance independently of concentration. The shapes of the curves obtained with protein preparations of types I and II show only minor differences.

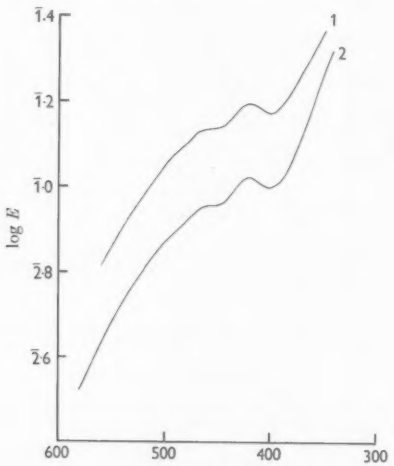

Fig. 6. Characteristic absorption spectra of pea-leaf-protein fractions active in catalysing TPN and metmyoglobin reduction. Curve 1, type II preparation further purified by electrophoresis on paper; curve 2, type I preparation.

Both types of preparation when stored at 0° progressively lost activity towards both TPN and metmyoglobin and this fall in activity was always accompanied by bleaching of the characteristic colour. There appeared to be a direct relationship between colour and activity but insufficient purified material has been available for this to be tested quantitatively.

DISCUSSION

It was stressed by Davenport & Hill (1960) that the important property of the 'haem–protein-reducing factor' is its specificity towards illuminated chloroplasts as donators of hydrogen. When metmyoglobin or cytochrome *c* were used as hydrogen acceptors in the photochemical reaction, they were regarded as convenient reagents for demonstrating the high activity of the leaf protein in catalysing hydrogen transport without reference to the nature of natural hydrogen acceptors in the green cell. The finding that TPN is even more effective than metmyoglobin as a hydrogen acceptor adds support to the suggestion (Davenport & Hill, 1960) that the protein 'could act as an important path for hydrogen transport in photosynthesis'. The important role of reduced TPN in carbon fixation is now well established and Arnon *et al.* (1958) go so far as to consider the light reaction in photosynthesis to consist essentially of the conversion of light energy into 'assimilatory power' in the form of reduced TPN and ATP.

From the observations reported above it would appear that, in spite of the very different fractionation procedures, the 'haem–protein-reducing factor' of Davenport & Hill (1960) is a more highly purified form of the 'photosynthetic pyridine nucleotide reductase' of San Pietro & Lang (1958). San Pietro (1958) has reported a further purification of his material from spinach leaves by using a Dowex-bentonite suspension to remove impurities from the protein recovered from the protamine complex. The final specific activity, with reference to TPN reduction, achieved by this step was 33·6 units/mg. of protein. In the present work, with pea leaves, the substitution of electrophoresis on paper for this final purification stage gave preparations having a specific activity up to 90 units/mg. of protein. Moreover, this product is apparently identical, both in activity towards TPN and metmyoglobin and in spectroscopic properties, with the protein obtained by the procedure of Davenport & Hill (1960).

An additional observation in the present work is the effect of a phosphate-acceptor system on the rates of TPN reduction which can be attained by

the use of increasing amounts of the active leaf protein of either type I or II. Rates of reduction up to 270 μmoles/mg. of chlorophyll/hr. have been obtained by including the complete phosphate-acceptor system. Calculated as the stoicheiometric equivalent amount of O_2 this value would correspond to $Q_{O_2}^{chl.}$ of 3000 μl. of O_2/mg. of chlorophyll/hr. This is equal to the maximum rate of photosynthesis for *Chlorella* reported by Hill & Whittingham (1955). In the absence of the phosphate-acceptor system the corresponding value of $Q_{O_2}^{chl.}$ did not exceed 800.

These results are in contrast to the report by Jagendorf (1959) that neither he, nor San Pietro, nor Arnon had been able to observe a stimulation of this kind. A possible explanation of these failures could be the use of an insufficient quantity of the catalytically active protein to raise the reduction rate to a level where the stimulatory effect can be observed (Fig. 2). Occasional failures to obtain the stimulation in the present work were traced to this cause and they usually arose with preparations of low specific activity when it was necessary to add relatively large volumes of the preparation in order to approach saturation of the particulate chloroplast system.

When this stimulation is considered, together with reports of Arnon *et al.* (1958) and San Pietro (1958) that TPN reduction can be coupled to photosynthetic phosphorylation by including the phosphate-acceptor system in the reaction mixture, the pattern of the reaction becomes analogous to that reported for ferricyanide reduction (Arnon *et al.* 1958, Avron *et al.* 1958). Here also the presence of a phosphate-acceptor system strongly stimulates hydrogen transport and concomitantly inorganic phosphate is incorporated into ATP. It therefore seems no longer necessary to postulate (Jagendorf, 1959) that, whereas ferricyanide reduction is fairly tightly coupled to phosphorylation, the coupling in TPN reduction is a loose one.

The capacity of a protein fraction to catalyse the reduction of some haem–protein compounds and TPN, but not DPN, introduces an unusual pattern of specificity if it is assumed that the purified material is a single homogeneous protein. No evidence of heterogeneity was obtained by boundary electrophoresis or in the ultracentrifuge (Davenport & Hill, 1960) and all subsequent attempts to separate the two activities by the more usual methods of protein fractionation have failed. The possibility, however, still remains that the purified material contains two proteins closely similar in their physical properties.

SUMMARY

1. A purified protein fraction from leaves, previously shown to be active in catalysing the reduction of some haem–protein compounds by illuminated chloroplasts (Davenport & Hill, 1960), will also catalyse the reduction of triphosphopyridine nucleotide (TPN) but not diphosphopyridine nucleotide.

2. Under appropriate conditions the rate of reduction of TPN but not that of metmyoglobin is stimulated up to threefold by including adenosine diphosphate, orthophosphate and magnesium ions in the reaction mixture. Arsenate will replace phosphate in producing this stimulation.

3. The reduction of metmyoglobin is progressively inhibited by TPN alone and by TPN with the phosphate-acceptor system present.

4. The protein obtained by Davenport & Hill (1960) has the properties of a more highly purified form of the photosynthetic pyridine nucleotide reductase of San Pietro & Lang (1958). It was found that the latter preparation is active in catalysing the reduction of metmyoglobin and could be further purified by electrophoresis on paper to give a product indistinguishable from that obtained by Davenport & Hill (1960).

REFERENCES

Arnon, D. I., Whatley, F. R. & Allen, M. B. (1958). *Science*, **127**, 1026.
Avron, M., Krogmann, D. W. & Jagendorf, A. T. (1958). *Biochem. biophys. Acta*, **30**, 144.
Bowen, W. J. (1949). *J. biol. Chem.* **179**, 239.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Colowick, S. P., Kaplan, N. O. & Ciotti, M. M. (1951). *J. biol. Chem.* **191**, 477.
Davenport, H. E. (1959*a*). *Nature, Lond.*, **184**, 524.
Davenport, H. E. (1959*b*). *Biochem. J.* **73**, 45P.
Davenport, H. E. & Hill, R. (1960). *Biochem. J.* **74**, 493.
Davenport, H. E., Hill, R. & Whatley, F. R. (1952). *Proc. Roy. Soc.* B, **139**, 346.
Hewitt, E. J. (1952). *Sand and Water Culture Methods.* Tech. Comm. 22. East Malling, England: Commonwealth Agricultural Bureau.
Hill, R. (1939). *Proc. Roy. Soc.* B, **127**, 192.
Hill, R. & Whittingham, C. P. (1955). *Photosynthesis.* London: Methuen and Co. Ltd.
Jagendorf, A. T. (1959). *Fed. Proc.* **18**, 974.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Mackinney, G. (1941). *J. biol. Chem.* **140**, 315.
Mazelis, M. (1956). *Plant Physiol.* **31**, 37.
San Pietro, A. (1958). *Brookhaven Symp. Biol.* **11**, 262.
San Pietro, A. & Lang, H. M. (1958). *J. biol. Chem.* **231**, 211.

Biochem. J. (1960) **77**, 478

The Reaction of Mustard Gas with Nucleic Acids *in vitro* and *in vivo*

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Mustard gas [di-(2-chloroethyl) sulphide] and analogous alkylating agents produce a wide range of biological effects (cf. review by Boyland, 1948). Some of these effects, such as induction of mutations (Auerbach & Robson, 1946) and interference with mitosis and chromosome breakage (Darlington & Koller, 1947), stimulated interest in the reactions of alkylating agents with deoxyribonucleic acid.

Previous studies of such reactions, mainly by physicochemical methods, have led to various suggestions regarding the chemical group or groups in nucleic acids involved. Chanutin & Gjessing (1946) showed by ultraviolet spectroscopy that the nitrogen mustards di-(2-chloroethyl)methylamine and tri-(2-chloroethyl)amine reacted with the bases of deoxyribonucleic acid, but the groups reacting were not specified. Elmore, Gulland, Jordan & Taylor (1948), on the basis of electrometric titration experiments, suggested that both basic and phosphate groups of deoxyribonucleic acid reacted with mustard gas. Press & Butler (1952) found that alkylation of deoxyribonucleic acid by di-(2-chloroethyl)methylamine in sodium bicarbonate solution resulted in a decrease of primary amino nitrogen and of purine nitrogen precipitable as purine silver salt, but no specific sites of reaction were established. Wheeler, Morrow & Skipper (1955) concluded from experiments with methylated xanthines and mustard gas that alkylation of ring nitrogen atoms of purines could occur. Alexander, Cousens & Stacey (1957) postulated that phosphotriesters were produced by alkylation of deoxyribonucleic acid in neutral aqueous solution by a variety of agents. This was based on observations that alkylation was not accompanied by liberation of acid. Reiner & Zamenhof (1957), from experiments on the binding of protamine by deoxyribonucleic acid and paper chromatography of hydrolysed alkylated deoxyribonucleic acid, claimed that whereas dimethyl sulphate reacted exclusively with the purine moieties at N-7, diethyl sulphate reacted exclusively with primary phosphate groups.

The only study of products formed by alkylation *in vivo* of nucleic acid is that by Wheeler & Skipper (1957). After administration of di-(2-chloroethyl)-[^{14}C]methylamine to rats and mice isotopic labelling of both ribonucleic acid and deoxyribonucleic acid was found, and it was shown that this was associated with the purine fraction.

In most of the earlier chemical studies of alkylation of nucleic acids the conditions were very different from those which would occur *in vivo*, e.g. large concentrations of alkylating agents, and sometimes alkaline pH, were used. Further, the methods used were not such as to enable the sites of reaction to be specified. In view of this uncertainty and the fact that preliminary experiments (Lawley & Wallick, 1957; Lawley, 1957*a*, *b*) had indicated N-7 of guanine moieties to be the most reactive centre towards alkylation in nucleic acid, it was decided to reinvestigate this reaction with a typical alkylating agent. Mustard gas, di-(2-chloroethyl) [^{35}S]sulphide, was chosen since it is known to be one of the most reactive of the alkylating agents and also is obtainable at a high specific radioactivity. The reaction of mustard gas with nucleic acid under mild conditions has therefore been studied chemically and compared with that resulting when tobacco mosaic virus, protoplasts of *Bacillus megaterium*, and the Ehrlich ascites tumour in the mouse are treated with this reagent.

EXPERIMENTAL

Materials. ^{35}S-Labelled mustard gas (about 30 mc/mmole) was obtained from The Radiochemical Centre, Amersham, Bucks. Ribonucleic acid (RNA) from rat liver and from yeast was supplied by Dr K. S. Kirby, deoxyribonucleic acid (DNA) from calf thymus by Professor J. A. V. Butler, protoplasts of *B. megaterium* by Dr G. D. Hunter and tobacco mosaic virus by Dr H. Fraenkel-Conrat. The Ehrlich ascites tumour was grown in stock mice in this Institute.

Paper chromatography and radioautography. Whatman no. 1 paper was used with the following solvents: methanol–ethanol–conc. HCl–water (50:25:6:19, by vol.) (solvent 1); butan-1-ol–conc. NH_3 soln.–water (86:1:13, by vol.) (solvent 2); ethanol–conc. NH_3 soln.–water (80:2:18, by vol.) (solvent 3); solvents (1) and (3) were run upward-flow and solvent (2) downward-flow. In two-dimensional paper chromatography solvent (1) followed by either solvent (2) or solvent (3) was used. Radioautographs were prepared by placing these papers, after drying, in contact with X-ray film in a light-tight container for up to 4 weeks. The lower limit of detection in this time was about 1 mμc per radioactive spot.

Counting techniques. Solid samples of nucleic acid and protein were plated at 'infinite thickness' on 1 cm.^{2} polythene planchets and counted with an end-window Geiger-Müller counter. The specific radioactivity of the samples was obtained by comparison with a ^{35}S-labelled standard of

known activity. Supernatants containing mustard gas and its hydrolysis products in aqueous ethanol were counted by adsorbing a known volume on to a weighed sample of charcoal which was then dried at room temperature under reduced pressure and plated in the usual way. When the amount of solid was insufficient for assay at 'infinite thickness', a sample of a solution of known concentration was evaporated on an aluminium planchet and counted at 'zero self-absorption' with a windowless gas-flow counter. Paper chromatograms of radioactive materials were assayed for radioactivity with a paper-strip counter incorporating a scintillation detector; in this apparatus 1 μmc gave 4·0 counts/sec. above background from an area of the paper of 6 cm. × 1 cm.

Reaction of mustard gas with guanosine. Guanosine (0·8 g., 2·8 m-moles) was dried at 100° and suspended in acetic acid (10 ml.). Mustard gas (0·55 ml., 4·6 m-moles) was added and the mixture heated at 100° for 2 hr. in a flask fitted with a drying-tube. After this time a clear solution resulted but on cooling a precipitate formed which was removed and shown to consist largely of unchanged guanosine. The filtrate was evaporated to dryness and the residue dissolved in N-HCl (15 ml.). After extraction of unchanged mustard gas with ether, this solution was heated at 100° for 1 hr., cooled and applied to a column of Dowex-50 (H^+ form, 22 cm. × 3 cm.). The column was developed with N-HCl and 50 ml. fractions were collected automatically; the ultraviolet absorption of these fractions was measured at 260 mμ. Fractions 40–55 contained guanine and fractions 75–120 contained a product of which the sum of the ultraviolet absorption at 260 mμ amounted to 30% of the total. These fractions were evaporated and excess of HCl was removed by further evaporation with water. The residue was dissolved in water and the solution neutralized with conc. NH_3 soln. The resultant precipitate was recrystallized from hot water to yield 7-(β-hydroxyethylthioethyl)guanine monohydrate (I) as clusters of colourless needles, m.p. decomp. above 280°. (Found: C, 39·9; H, 5·5; N, 25·5; loss at 150°, 5·8. $C_9H_{13}O_2N_5S,H_2O$ requires C, 39·6; H, 5·5, N, 25·7; H_2O, 6·6%. After drying *in vacuo* at 150°; Found: C, 42·4; H, 4·8. $C_9H_{13}O_2N_5S$ requires C, 42·5; H, 4·7%). Ultraviolet spectra and R_F values are given in Table 1.

Subsequent elution of the column with 4 N-HCl gave a product (III) in about 5% yield which could not be crystallized but had similar ultraviolet spectra (Table 1).

7-(*β-Hydroxyethyl[^{35}S]thioethyl)guanine* was prepared in a similar experiment with ^{35}S-labelled mustard gas (200 mg., 6 mc). The specific radioactivity of the product was 16·5 μc/mg.

Reaction of 7-(β-hydroxyethylthioethyl)guanine with hydrogen peroxide. 7-(β-Hydroxyethylthioethyl)guanine (53 mg.) was dissolved in 0·25 N-HCl (1·3 ml.), 100 vol. H_2O_2 (0·2ml.) added and the mixture was stood at room temp. for 2 hr. The solution was neutralized with conc. NH_3 soln. and the resultant precipitate recrystallized from water as clusters of colourless needles. Yield, 26 mg., m.p. decomp. above 265° (Found: C, 37·6; H, 4·8; N, 24·4; S, 10·6. $C_9H_{12}O_4N_5S$ requires C, 37·6; H, 4·5; N, 24·4; S, 11·1%).

Reaction of mustard gas with ribonucleic acid and deoxyribonucleic acid in vitro. RNA or DNA (150 mg.) was dissolved in mM-potassium acetate pH 7·2 (30 ml.) at 37° and ^{35}S-labelled mustard gas (46·7 μg., 1 m-mole/mole of nucleic acid P, in 0·01 ml. of ethanol) was added. After 1, 2, 3, 5 min. and 20 hr., samples (6 ml.) were taken and nucleic acid was precipitated with 2 vol. of ethanol containing 2% potassium acetate. The washed and dried nucleic acid and supernatant fluids were assayed for radioactivity as described above and radioautographs prepared from two-dimensional paper chromatograms.

In other experiments where larger amounts of mustard gas were used the concentration of acetate was 4 moles/mole of mustard gas.

Hydrolysis of nucleic acid samples. RNA was hydrolysed with N-HCl (Smith & Markham, 1950). DNA, and the mixed nucleic acids from *B. megaterium* were hydrolysed with formic acid (Wyatt, 1951) to obtain base ratios by ultraviolet spectroscopy; to obtain chromatograms for radioautography incomplete hydrolysis with N-HCl at 100° for 1 hr. was used.

Reaction of mustard gas with tobacco mosaic virus. Tobacco mosaic virus (47·5 mg.) was suspended in 0·1 mM-$Na_4P_2O_7$ buffer, pH 7·0 (4·75 ml.) at 37° and ^{35}S-labelled mustard gas (6 μg., 0·375 μc) in ethanol (0·01 ml.) was added. In a similar experiment 1·2 μg. of ^{35}S-labelled mustard gas was used. After 45 min. the suspension was centrifuged at 100 000*g* for 15 min., the tobacco mosaic virus washed by resuspension and recentrifuged. For isolation of RNA and protein the suspension of tobacco mosaic virus in water (1 ml.) was shaken with 90% (w/w) phenol (1 ml.) for 1 hr. and the layers separated by centrifuging at 20 000*g*. RNA was precipitated from the aqueous layer with ethanol (2 vol.) containing 2% (w/v) sodium acetate, and protein from the phenol layer with a mixture of methanol (5 vol.) and ether (10 vol.). RNA was assayed for radioactivity at 'zero self-absorption' and protein at 'infinite thickness'. Radioautographs were prepared from two-dimensional paper chromatograms of hydrolysed RNA.

Treatment of protoplasts of B. megaterium *with mustard gas. B. megaterium* (500 mg.) was converted into protoplasts, suspended in 'C-phosphate' (60 ml.) (Hunter, Brookes, Crathorn & Butler, 1959) and incubated at 30° for 1 hr. before addition of [^{35}S]mustard gas (6·7 mg., 0·9 mc) in ethanol (0·5 ml.). After a further incubation for 90 min. protoplasts were sedimented and homogenized in 2·5% (w/v) sodium salicylate (20 ml.). Nucleic acid and protein were isolated by the phenol method as for tobacco mosaic virus, above, and assayed for radioactivity. A sample of nucleic acid was hydrolysed with formic acid and a radioautograph prepared of a two-dimensional chromatogram.

Treatment of Ehrlich ascites tumour with mustard gas in vitro. Ehrlich ascites tumour cells were obtained from 7-day-old ascites tumours grown by transplantation in stock mice. The cells were sedimented and washed twice with 0·9% NaCl; 3×10^8 cells were suspended in Hank's medium (Hank & Wallace, 1949) (10 ml.) at 37° and ^{35}S-labelled mustard gas (0·2 mg., 36·2 μc) was added. Samples (2 ml.) were removed after 1, 5, 15, 30 and 60 min., diluted with ice-cold 0·9% NaCl (10 ml.), sedimented, washed twice with ethanol (2 ml.), resedimented, dried and assayed for radioactivity at 'infinite thickness'.

In a similar experiment $1·7 \times 10^9$ cells were suspended in Hank's medium (37 ml.) and treated with ^{35}S-labelled mustard gas (2·67 mg., 440 μc). RNA (23 mg.) was isolated by the method of Colter & Brown (1956) and assayed for radioactivity; a sample was hydrolysed and a radioautograph prepared in the usual way.

Treatment of Ehrlich ascites tumour with mustard gas in vivo. Five mice bearing 7-day-old Ehrlich ascites tumours were each injected with ^{35}S-labelled mustard gas (in one series 10 μg., 1·1 μc; in others 30 μg., 2·8 μc; 100 μg., 16 μc) in arachis oil (0·1 ml.). After 30 min. the mice were killed and RNA and protein were isolated from the ascites cells as described above. DNA was isolated from the nuclear material insoluble in 0·14 M-NaCl by the method of Kirby (1959), omitting the ribonuclease treatment and the separation from polysaccharide. [The RNA had 7·5% of P; ϵ_P (extinction/mole of nucleic acid P) in 0·01 N-sodium phosphate pH 7, 7000; molar base ratios, adenine, 1; guanine, 1·89; cytosine, 1·52; uracil, 1·07. DNA had 7·5% of P; ϵ_P in 0·01 N-sodium phosphate pH 7, at 20°, 6800; after 15 min. at 100°, 8800; molar base ratios, adenine, 1: guanine, 0·78; cytosine, 0·75; thymine, 0·92; uracil, 0·05]. The presence of uracil in the DNA shows that RNA was present as an impurity to the extent of about 5%. This results from omission of ribonuclease treatment in order to avoid prolonged dialysis of the DNA with consequent loss of alkylated guanine. Samples of nucleic acid and protein were assayed for radioactivity and radioautographs were prepared in the usual way.

RESULTS

The reaction of mustard gas with both RNA and DNA with a low molar proportion of ^{35}S-labelled mustard gas to nucleic acid P at 37° and neutral pH was found to occur rapidly and extensively (Fig. 1). In the initial stages the rate of reaction was the same for both RNA and DNA but differences appeared at later times in that a large part of the radioactivity, initially bound to DNA, was released into the supernatant.

Radioautographs of two-dimensional paper chromatograms of hydrolysed [^{35}S]mustard gas-treated nucleic acids were very similar and showed three major radioactive spots (Fig. 2).

In experiments with a higher molar proportion of mustard gas (0·3 mole/mole of nucleic acid P) these products were obtained in amounts sufficient for measurement of their ultraviolet-absorption spectra after elution from the paper. The spectra of (I), (II) and (III) (Fig. 2) were virtually identical and

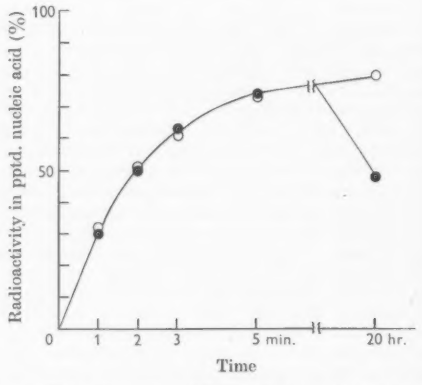


Fig. 1. Rate of reaction of ^{35}S-labelled mustard gas with RNA (yeast) and DNA (calf thymus). The reaction mixture contained 1 m-mole of mustard gas/mole of nucleic acid P in neutral aqueous solution at 37°. ○, RNA; ●, DNA.

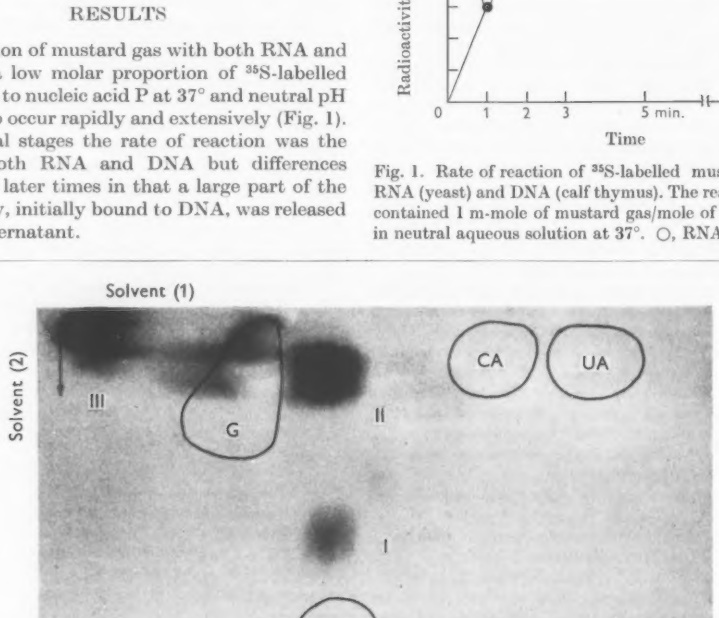


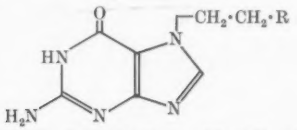
Fig. 2. Typical radioautograph of a two-dimensional paper chromatogram of hydrolysed RNA after reaction with ^{35}S-labelled mustard gas (1 m-mole/mole of RNA P). The positions of unchanged bases of RNA on the paper chromatogram, as shown, were determined by examination of the paper under ultraviolet light (λ, 2537 Å). A, Adenine; G, guanine; CA, cytidylic acid; UA, uridylic acid. Solvent (1), methanol:ethanol:HCl:water. (50:25:6:19, by vol.); solvent (2), butan-1-ol:conc. NH_3:water, (86:1:13, by vol.).

Table 1. *R_F values and ultraviolet-absorption spectra of products of alkylation of nucleic acid by mustard gas*

Product	R_F			$\lambda_{max.}$ (mμ)		
	Solvent (1)	Solvent (2)	Solvent (3)	pH 1	pH 7	pH 12
(I)	0·37	0·20	0·45	250 (ϵ 11 400)	284 (ϵ 7400)	281 (ϵ 7300)
(II)	0·37	0·04	0·20	250	284	281
(III)	0·03	0·0	0·0	250	284	281
(IV)	0·14	0·04	0·20	250	284	281

closely resembled those of 7-methylguanine (Lawley, 1957*a*), (Table 1).

The reaction of guanosine and mustard gas was therefore investigated by column chromatography and yielded two products. The major product was purified and analysed for 7-(β-hydroxyethylthioethyl)guanine; the minor product, which could be eluted from the column only with concentrated acid, could not be purified but its behaviour on paper chromatography was identical with that of (III). The major product on paper chromatography ran mainly with R_F values identical with those of (I), but always gave a small proportion of a second component having the same R_F in solvent (1) but lower R_F in solvents (2) and (3). The R_F values of this second component were identical with those of (II) (Table 1), and further when (I) was eluted from the paper and re-run partial conversion into (II) was found. It appears, therefore, that (I) is 7-(β-hydroxyethylthioethyl)guanine and that (II) is a closely related substance formed from it during paper chromatography. When (II) was eluted from the paper and re-run it was partially converted into



R = $-S\cdot CH_2\cdot CH_2\cdot OH$ (I); $-SO_2\cdot CH_2\cdot CH_2\cdot OH$ (IV)

a product (IV) with lower R_F in solvent (1). A substance with the chromatographic properties of (IV), probably 7-(β-hydroxyethylsulphonylethyl)-guanine, was obtained when (I) was treated with hydrogen peroxide under mild conditions; the ultraviolet-absorption spectra of this substance were again identical with those of (I). It appears, therefore, that modification of the side chain of (I) occurs readily under mild conditions, such as those of paper chromatography, and the experiments with hydrogen peroxide suggest that oxidation may be involved. When very small amounts of these products were present on the paper chromatograms, as with experiments *in vivo*, the proportion of (II) relative to (I) was higher, with (II) sometimes predominating.

Table 2. *Specific radioactivity of ribonucleic acid and deoxyribonucleic acid*

RNA (rat liver) and DNA (calf thymus) were treated with ^{35}S-labelled mustard gas (1 m-mole/mole of nucleic acid P) at pH 7, and 37° for the time stated, before isolation of nucleic acid by precipitation. Specific radioactivity of solid nucleic acid was obtained at 'infinite thickness', and was also calculated from strip-counting of radioactive spots on paper chromatograms of hydrolysed samples of nucleic acid.

	RNA	RNA	DNA	DNA
Time (min.)	1	15	1	15
Specific radioactivity of solid (μc/g.)	36	66	18	37
Specific radioactivity from paper chromatogram (μc/g.)	42	89	17	37

Since the products show a degree of instability, the effect of conditions used for hydrolysis of nucleic acids on 7-(β-hydroxyethyl[^{35}S]thioethyl)-guanine was investigated. N-Hydrochloric acid at 100° for 1 hr. had no effect, but formic acid at 175° for 1 hr. caused partial decomposition of this compound with formation of guanine, indicated by paper chromatography, and of isotopically-labelled products which ran at the solvent front in solvent (1). These products are probably derived from the aliphatic side chain since mustard gas and its products of hydrolysis in aqueous solution behave similarly.

As shown in Fig. 1 the radioactivity of DNA treated with ^{35}S-labelled mustard gas rapidly reaches a maximum and then decreases slowly, and this is accompanied by a decrease in the viscosity of the solution. The radioactivity which is lost appears in the supernatant fluid when the DNA is precipitated, and paper chromatograms of the supernatant showed it to contain products (I), (II) and (III). When DNA was treated with 0·3 mole of mustard gas/mole of P a precipitate began to form after about 20 hr. This was isolated and paper chromatography showed it to be mainly (III) together with traces of (I) and (II).

The extent of the reaction between ^{35}S-labelled mustard gas and nucleic acid was estimated from the specific radioactivity of the isolated treated nucleic acid. It was also estimated by assaying the

Table 3. *Amount of reaction of* ^{35}S-*labelled mustard gas with tobacco mosaic virus* B. megaterium *protoplasts and Ehrlich ascites tumour in the mouse*

Tobacco mosaic virus and *B. megaterium* protoplasts were treated in buffer for the time stated. Ehrlich ascites tumour was treated by injection of tumour-bearing mice with ^{35}S-labelled mustard gas in arachis oil, the mice being killed after the time stated. The figure for dose given to mice is based on an average weight of 50 g./mouse. The figure for the amount of mustard gas that reacted with nucleic acid is based on an average P content for nucleic acid of 7·5%. Nucleic acid from *B. megaterium* was about 70% RNA and 30% DNA.

	Time (min.)	Dose of [^{35}S]mustard gas (μc/g.)	Mustard gas found in whole virus or whole cells (% of dose)	Specific radioactivity (μc/g.)			Amount of mustard gas which reacted with nucleic acid (μmoles/mole of nucleic acid P)	
				Protein	RNA	DNA	RNA	DNA
Tobacco mosaic virus	45	7·9	37	2·8	4·9	—	248	—
Tobacco mosaic virus	45	1·5	47	0·7	0·8	—	40	—
B. megaterium	90	1750	1	22	31	—	700	—
Tumour	15	0·16	—	—	0·6	0·6	20	20
Tumour	30	0·32	—	1·9	2·4	2·2	40	37
Tumour	70	0·06	1·8	0·30	0·25	0·25	7	7
Tumour	30	0·02	1·2	0·09	0·08	0·09	2·4	2·7

radioactivity on a two-dimensional paper chromatogram of hydrolysed nucleic acid with a paper-strip counter. The amount of nucleic acid on the paper was determined by eluting the adenine spot and measuring its ultraviolet absorption. The estimates by the two methods were in good agreement (Table 2), thus confirming that the products on the paper chromatograms accounted for the whole of the reaction.

In order to ascertain the effect of excess of protein and other cellular constituents as competitors of nucleic acid in reaction with ^{35}S-labelled mustard gas the reaction with tobacco mosaic virus and *B. megaterium* was studied. In both cases the specific radioactivity of nucleic acid was greater than that of protein (Table 3). Radioautographs of paper chromatograms, obtained in the usual way, showed that the reaction with nucleic acid was of the characteristic type described above.

Since mustard gas is known to be an inhibitor of tumour growth (Bass & Freeman, 1946) it was of interest to study its reaction with tumour cells, and the Ehrlich ascites tumour was chosen since the cells show a high degree of uniformity and are readily available. A study *in vitro* showed that mustard gas was taken up rapidly by the whole cells, attaining a maximum of 12% of the given amount after 30 min., and that the isolated RNA had reacted in the characteristic way. In further experiments, mice bearing the tumour were injected with ^{35}S-labelled mustard gas and the RNA, DNA and protein were subsequently isolated from the tumour cells. The results were similar to those obtained *in vitro* except that a lower proportion of the mustard gas administered was taken up by the tumour cells (Table 3). The specific radioactivities of the RNA and DNA were approximately equal. To avoid loss of alkylated guanine from DNA during its isolation, traces of RNA and polysaccharide were not removed since this would have involved prolonged dialysis. The analytical data for the nucleic acids showed that the amount of impurity was small.

DISCUSSION

The present work, which was designed to establish the nature of the reaction of a low concentration of mustard gas with nucleic acid, when only the most reactive centres would be attacked, has clearly established that alkylation of guanine is the only reaction occurring in detectable amount. The technique used enabled the reaction products to be identified when the extent of the reaction was about 30 μmoles of mustard gas reacting/mole of nucleic acid phosphorus. This level of reaction was that which occurred with the nucleic acid of the Ehrlich ascites tumour in the mouse after injection of 0·1 mg. of mustard gas.

Earlier work (Butler, Gilbert, James & Ross, 1951) had suggested that esterification of phosphate groups might be an important reaction of alkylating agents with nucleic acids. If this had occurred to any extent in the case of mustard gas, hydrolysis of partially esterified nucleic acid would be expected to result in the formation of simple hydrolysis products of mustard gas, but these were not found in the present investigation.

Alkylation of guanine moieties of nucleic acids

also accounts for the observed difference in behaviour between RNA and DNA after alkylation, namely that whereas alkylated RNA appears to be stable in neutral aqueous solution at 37°, alkylated DNA decomposes with loss of 7-alkylguanine, since it has been shown (Lawley, 1957*a*) that methylated deoxyguanylic acid hydrolyses at the deoxyriboside linkage under these conditions. On the other hand, esterification of phosphate groups of nucleic acids to give phosphotriester groups would be expected to result in destabilization of RNA but not of DNA, since it has been shown (Brown, Magrath & Todd, 1955) that dimethyl and dibenzyl uridine 3′-phosphate are unstable at pH 7 and 37°. This instability was attributed to the formation of an unstable intermediate by a cyclization involving the phosphorus atom and the oxygen of the vicinal hydroxyl of ribose. As the deoxyribose moiety of DNA has no free hydroxyl group such an intermediate could not be formed, and therefore phosphotriester groups in DNA would be expected to have the stability normally associated with a trialkyl phosphate (Brown *et al.* 1955).

The suggestion (Butler *et al.* 1951) that esterification of phosphate groups might occur on alkylation of nucleic acids by compounds of the mustard type appears to be supported by observations that alkylation at pH 7 occurs without liberation of acid (Stacey, Cobb, Cousens & Alexander, 1958). These latter authors stated that this evidence showed unambiguously that alkylation of DNA *in vivo* would be confined entirely to esterification. They appear not to have considered the fact that alkylation of ring nitrogen atoms to yield quaternary ammonium derivatives would result in the same experimental finding.

The experiments of Press & Butler (1952) showing that alkylation of nucleic acid by di-(2-chloroethyl)methylamine caused a decrease in the amount of purine nitrogen precipitable by silver and in purine amino nitrogen might appear to be in disagreement with the present findings that N-7 of guanine moieties is the site of alkylation. However, their experiments used alkaline conditions under which it is known that the product of alkylation at this site would be unstable (Lawley & Wallick, 1957), yielding a product non-precipitable by silver. Further, the excess of alkylating agent used would be expected to result in alkylation at less reactive sites, such as N-1 or N-3 of adenine (Brookes & Lawley, 1960), with consequent loss of primary amino nitrogen.

It has been suggested, on the basis of physicochemical evidence, that alkylation of DNA results initially in its partial aggregation (Elmore *et al.* 1948; Alexander & Stacey, 1958; Bendich, di-Mayorca, Rosoff & Rosenkranz, 1959), but with a subsequent decrease in its molecular weight (Conway, Gilbert & Butler, 1950). The present work does not establish chemical mechanisms which account for these observations but the product (III) has properties which are not inconsistent with its having resulted from linkage, by mustard gas, of two guanine moieties, which may have been derived from different molecular chains of DNA. Subsequent decrease in the molecular weight of alkylated DNA might follow the loss of 7-alkylguanine since a mechanism for the fission of the DNA molecular chain after such a loss of a purine moiety has been suggested (Brown & Todd 1955).

The studies reported here of the reaction of mustard gas with tobacco mosaic virus, *B. megaterium* and the Ehrlich ascites tumour have established that the presence of large excess of protein and other cellular constituents does not prevent the alkylation of nucleic acid, in agreement with similar findings in other systems (Butler, 1956). Further, the experiments with the Ehrlich ascites tumour in the mouse show that the same reaction occurs *in vivo*. The amount of ^{35}S-labelled mustard gas taken up by the tumour cells *in vivo* was small (Table 3) but as usual the specific radioactivity of nucleic acid was about the same as that of the protein or of the whole cells. The extent of reaction with tumour nucleic acid after the injection of tumour-bearing mice with 0·1 mg. of mustard gas/mouse can be expressed as about 1 molecule of mustard gas/molecule of DNA of molecular weight 6×10^6. This dose of mustard gas is of the order of that shown to inhibit growth of certain mouse tumours (Bass & Freeman, 1946). Tests of mustard gas against the Ehrlich ascites tumour do not appear to have been reported, but the nitrogen mustard di-(2-chloroethyl)methylamine is an effective inhibitor of growth of this tumour at a dose of 0·2 mg./kg. (Creech, 1958).

SUMMARY

1. A study has been made of the reaction of ^{35}S-labelled mustard gas with nucleic acid in neutral aqueous solution at 37° with a low concentration of mustard gas.

2. The only detectable reaction with both ribonucleic acid and deoxyribonucleic acid was at N-7 of guanine moieties, the hydrolysis products having chromatographic properties identical with those obtained from guanosine and mustard gas.

3. The principal product from nucleic acid and mustard gas, 7-(β-hydroxyethylthioethyl)guanine, has been synthesized.

4. Ribonucleic acid alkylated by mustard gas is stable in neutral aqueous solution but alkylated deoxyribonucleic acid decomposes with loss of 7-alkylguanine.

5. Tobacco mosaic virus, *Bacillus megaterium* and cells of the Ehrlich ascites tumour have been treated with ^{35}S-labelled mustard gas; the extent of reaction with nucleic acid has been determined and shown to be about the same as that with protein. The site of reaction with nucleic acid has been shown, as in the case of pure nucleic acid, to be at N-7 of guanine moieties.

6. Mice bearing the Ehrlich ascites tumour have been injected with ^{35}S-labelled mustard gas and the nucleic acid of the tumour cells shown to have reacted in the same way as *in vitro*.

7. Previous work on the reactions of alkylating agents with nucleic acids is discussed in relation to the present findings.

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REFERENCES

Alexander, P., Cousens, S. F. & Stacey, K. A. (1957). In *Ciba Foundation Symp., Drug Resistance in Microorganisms*, p. 294. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. and A. Churchill Ltd.

Alexander, P. & Stacey, K. A. (1958). *Ann. N.Y. Acad. Sci.* **68**, 1225.

Auerbach, C. & Robson, J. M. (1946). *Nature, Lond.*, **157**, 302.

Bass, A. D. & Freeman, M. L. H. (1946). *J. nat. Cancer Inst.* **7**, 171.

Bendich, A., diMayorca, G., Rosoff, M. & Rosenkranz, H. S. (1959). *Trans. Faraday Soc.* **55**, 491.

Boyland, E. (1948). *Symp. biochem. Soc.* **2**, 61.

Brookes, P. & Lawley, P. D. (1960). *J. chem. Soc.* p. 539.

Brown, D. M., Magrath, D. I. & Todd, A. R. (1955). *J. chem. Soc.* p. 4396.

Brown, D. M. & Todd, A. R. (1955). In *The Nucleic Acids*, vol. 1, p. 444. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.

Butler, J. A. V. (1956). In *Ciba Foundation Symp., Ionizing Radiations and Cell Metabolism*, p. 67. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. and A. Churchill Ltd.

Butler, J. A. V., Gilbert, L., James, D. W. F. & Ross, W. C. J. (1951). *Nature, Lond.*, **168**, 985.

Chanutin, A. & Gjessing, E. C. (1946). *Cancer Res.* **6**, 599.

Colter, J. S. & Brown, R. A. (1956). *Science*, **124**, 1077.

Conway, B. E., Gilbert, L. & Butler, J. A. V. (1950). *J. chem. Soc.* p. 3421.

Creech, H. J. (1958). *Ann. N.Y. Acad. Sci.* **68**, 868.

Darlington, C. D. & Koller, P. C. (1947). *Heredity*, **1**, 187.

Elmore, D. T., Gulland, J. M., Jordan, D. O. & Taylor, H. F. W. (1948). *Biochem. J.* **42**, 308.

Hank, J. H. & Wallace, R. E. (1949). *Proc. Soc. exp. Biol., N.Y.*, **71**, 196.

Hunter, G. D., Brookes, P., Crathorn, A. R. & Butler, J. A. V. (1959). *Biochem. J.* **73**, 369.

Kirby, K. S. (1959). *Biochim. biophys. Acta*, **36**, 117.

Lawley, P. D. (1957*a*). *Proc. chem. Soc., Lond.*, p. 290.

Lawley, P. D. (1957*b*). *Biochim. biophys. Acta*, **26**, 450.

Lawley, P. D. & Wallick, C. A. (1957). *Chem. & Ind.* p. 633.

Press, E. M. & Butler, J. A. V. (1952). *J. chem. Soc.* p. 626.

Reiner, B. & Zamenhof, S. (1957). *J. biol. Chem.* **228**, 475.

Smith, J. D. & Markham, R. (1950). *Biochem. J.* **46**, 509.

Stacey, K. A., Cobb, M., Cousens, S. F. & Alexander, P. (1958). *Ann. N.Y. Acad. Sci.* **68**, 682.

Wheeler, G. P., Morrow, J. S. & Skipper, H. E. (1955). *Arch. Biochem. Biophys.* **57**, 133.

Wheeler, G. P. & Skipper, H. E. (1957). *Arch. Biochem. Biophys.* **72**, 465.

Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.

Biochem. J. (1960) **77**, 484

The Simple Monosubstituted Guanidines of Mammalian Brain

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Although observations have been made on individual monosubstituted guanidine derivatives of the brain, there appears to have been no systematic investigation of their total amount or number. Such a study has now been performed. Values for the concentration of total monosubstituted (Sakaguchi-positive) guanidines have been obtained, in guinea-pig, ox and rat brain; paper-chromatographic studies have confirmed the presence of arginine, glycocyamine (guanidoacetic acid) and γ-guanidobutyric acid, and have indicated the occurrence of taurocyamine (guanidoethane sulphonic acid). Approximate values for concentrations of the individual bases have also been obtained.

METHODS

Materials. Commercial arginine and glycocyamine were recrystallized as the hydrochlorides; commercial creatine was purified with charcoal and recrystallized from water (King, 1930). γ-Guanidobutyric acid was prepared from cyanamide (Engeland & Kutscher, 1910) and recrystallized as the hydrochloride (m.p. 184°, corr.). Taurocyamine was prepared from *iso*thiourea (Schütte, 1943; Morgue, 1948; Van Thoai & Robin, 1954); recrystallization of the free base from water, ethanol or ethanol–acetone yielded white crystals, melting sharply at 267° uncorr. (Engel, 1875; Morrison, Ennor & Griffiths, 1958). Both synthetic products chromatographed identically, in six solvents, with samples of the authentic compounds kindly given by Dr I. Smith.

Commercial 8-hydroxyquinoline was recrystallized from ethanol (m.p. 72–73°, corr.). Chromatographic solvents were of chromatography grade where available or else analytically pure. Other reagents were analytic grade unless specified otherwise.

Rats and guinea pigs were obtained commercially; no attempt was made to control strain or sex differences. Ox brain was obtained fresh at a slaughter house.

Paper chromatography. Descending chromatograms were developed at room temperature. Standard solvents were butan-1-ol–acetic acid–water (12:3:5, by vol.) and butan-1-ol–pyridine–water (1:1:1, by vol.); in two-dimensional chromatography the acidic solvent was used first (Smith, 1958). Other solvents were: propan-2-ol–NH_3 soln. (sp.gr. 0·88)–water (73:4:23, by vol.) (Robin, 1954); propan-2-ol–acetic acid–water (15:1:4, by vol.) and butan-1-ol–formic acid–water (77:11:12, by vol.). On Whatman no. 1 chromatography paper, unwashed, up to 2 mg. could be applied on a single spot. On Whatman no. 3MM paper, used for larger quantities, the bases ran more rapidly and diffusely. Compounds were detected by spraying with Sakaguchi reagents, which clearly show 2 μg. of monosubstituted guanidines and give no colour even with 2·5 mg. of creatine (Smith, 1958). For creatine, pentacyanoferriate spray was used (Smith, 1958). R_F values in the first three solvents were as described by Smith (1958) and Robin (1954); values in the other two are summarized in Table 1. Although the temperature was not controlled, variations in R_F tended to be small (S.D. about 10%). In particular experiments values up to ±25% from the average occurred with pure compounds and impure extracts; however, controls, run on all chromatograms, retained their relative positions as discrete spots. Recoveries of the bases on elution with N-HCl were 80–100% (four determinations each on arginine, glycocyamine and γ-guanidobutyric acid).

Assay

Monosubstituted guanidines were estimated by the modification of the Sakaguchi reaction of Dubnoff (1941, 1957), in which up to 0·2 mg. of creatine or creatinine gave an insignificant colour; greater quantities gave irregular but significant readings.

With precise control, and rapid, reproducible addition of hypobromite the reaction is quantitative in the range 0·02–0·15 μmole. In 100 determinations in various extracts over the course of a year, the average difference between duplicates was 4±4% (S.D.): recoveries from tissue extracts were quantitative (see below). The bases have different extinction coefficients (Table 2). Arginine, known to be present in relatively large amounts (see Discussion), was chosen as colorimetric standard and values are expressed as μmoles of arginine.

Tissue extracts

Removal of tissue. Usually rats or guinea pigs were killed by a blow on the neck, the brain was removed, blotted on Whatman no. 50 paper and either ground immediately in deproteinizing agent in a tared, all-glass homogenizer or rapidly weighed and then fixed. Time from blow to fixing was no more than 3·5 min.; no difference caused by the two methods of obtaining weight was noted. Usually cerebral hemispheres and cerebellum were used; when comparison with frozen material was needed, only cerebral hemispheres were taken.

In one series of experiments, young rats (2 weeks, 100 g.) were dropped head-first into liquid nitrogen and the cerebral hemispheres were chiselled out (Richter & Dawson, 1948; McIlwain, Buchel & Cheshire, 1951). Samples of tissue allowed to come to room temperature contained, on examination, no bone.

Ox brain, removed at the slaughter house, was transported to the laboratory at ice-bath temperature.

Deproteinization. Unless described otherwise, homogenates were prepared at ice-bath temperature and centrifuged for 20 min. at 10 000 *g* and 0°±2°. In calculating tissue concentrations from portions of the supernatant, the tissue was assumed to contribute a volume of water equal to 80% of its wet weight to the homogenate.

Table 1. *R_F values of guanidine bases*

Chromatograms were developed at room temperature, on Whatman no. 1 chromatography paper, and spots were detected with either Sakaguchi or pentacyanoferriate spray reagents (see text). Figures represent the means of at least four determinations; S.D. is about 10%.

	R_F	
Base	Solvent: butanol–formic acid–water (77:11:12 by vol.)	Solvent: propan-2-ol–acetic acid–water (15:1:4 by vol.)
Arginine	0·07	0·17
γ-Guanidobutyric acid	0·45	0·56
Glycocyamine	0·23	0·38
Taurocyamine	0·11	0·29
Creatine	0·35	0·33

Table 2. *Extinction coefficients of guanidine bases in Sakaguchi reaction*

Method of Dubnoff was used (see text). The value of the extinction coefficient is followed by the S.D. and the number of duplicate determinations. The μmolar extinction coefficient is given.

Base	$10^6\,\epsilon$
Arginine hydrochloride	4·6±0·6 (28)
γ-Guanidobutyric acid hydrochloride	6·4±0·6 (8)
Glycocyamine hydrochloride	5·5±0·6 (6)
Taurocyamine (free base)	9·5±0·1 (4)

Extracts with trichloroacetic acid (10%, w/v) were prepared by grinding brain (0·2–0·4 g./ml.) in the acid and centrifuging. Assay of the clear to slightly opalescent supernatant showed no dilution effects: there was no systematic variation with different sized portions or different concentrations of tissue in deproteinizing agent.

Extracts with tungstic acid were prepared by grinding the tissue (0·2–0·4 g./ml.) in fresh, dilute, phosphate-stabilized tungstic acid reagent (Caraway, 1958) and centrifuging. Since material interfering with the Sakaguchi assay was extracted by tungstic acid (Van Pilsum, Martin, Kito & Hess, 1957), two different-sized samples of the supernatant were taken (e.g. 0·5 and 1·0 ml.) and the results were extrapolated to zero-tissue concentration (e.g. the difference between concentration calculated from the 0·5 ml. and from the 1·0 ml. samples was added to the concentration calculated from the 0·5 ml. sample).

In preparing metaphosphoric acid extracts, all solutions of the reagent must be kept cold (0°–5°). A stick of metaphosphoric acid ($NaPO_3$-stabilized) was thoroughly washed, covered with enough water to make a 20% solution and left overnight. This solution was shaken and diluted to 6% (w/v); brain (up to 0·2 g./ml.) was ground in dilute reagent and centrifuged. The supernatant was clear but foamed on shaking.

The following procedure, performed as rapidly as possible, was adopted for deproteinization by heating (Hunter, 1957). A homogenizer and pestle containing 20 ml. of water were placed in a boiling-water bath (95°–99°). Brain (up to 4 g.) was weighed to the nearest 0·1 g., the homogenizer was removed from the bath, 0·3 m-equiv. of acetic acid/g. of brain was added to the water, the tissue was homogenized in the hot dilute acid and the homogenate (pH about 5·8) was returned to the boiling-water bath for 10 min. Material tended to clot at the top and bottom of the suspension. Centrifuging gave a clear to slightly opalescent supernatant which could, with care, be removed by Pasteur pipette from the very soft precipitate. Ox brain was deproteinized by blending the tissue (top-drive macerator) in 3–5 vol. of hot dilute acetic acid (80°–90°, 3 m-equiv. of acetic acid/g. of tissue), and boiling the suspension for 10 min. and centrifuging for 15 min. at 1400 *g* at room temperature. Supernatants were cloudy and foamy.

Purification of extracts for paper chromatography

Initial observations of the presence of guanidine derivatives in cerebral extracts were made by B. M. Bollard & H. McIlwain (unpublished work) during a study of cerebral purines.

Purification on ion-exchange resin. The alkaline constituents of tissue extracts prepared with tungstic acid from about 5 g. of brain were concentrated on Amberlite IR-120(H) (100–200 mesh), which was prepared by alternately washing with 5N-HCl (three times) and with 2N-NaOH (twice) and stored dry in the H^+ form. Eluents with NH_3 soln. of thoroughly washed resin gave no Sakaguchi reaction on paper but reacted irregularly in the assay, corresponding to 0·3–3·0 μg. of arginine/ml. of eluent.

The 60–70% of the tungstic acid supernatant which could be removed (by Pasteur pipette) from the precipitate was added to a column of resin (2·5 g./g. of tissue; height: diameter > 9:1). The column, run at 0·5 ml./min., was washed with water (20 ml./g. of tissue) and with 2N-NH_3 soln. (25 ml./g. of tissue). The water eluate was discarded and the ammonia eluate was vacuum-distilled to dryness and taken up in two 2 ml. portions of N-HCl; a white insoluble residue was removed by gravity filtration (Whatman no. 43 paper), and the filtrate evaporated to dryness on the rotary evaporator (Aves, 1959); the residue was dissolved in 2 ml. of N-HCl/mg. of guanidine. Quantitative estimates of recovery are prevented by the variable resin blank; assay suggested recovery from the column of 80–90%, recovery from the tissue of 50% and a 50-fold concentration of monosubstituted guanidines (to 2% of non-protein dry weight).

In four control experiments, creatine (1·5 mg./g. of resin) was taken through the procedure; chromatograms showed no material reacting with the Sakaguchi reagents.

Large-scale preparations. Extracts of about 0·5 kg. of ox brain which had been deproteinized by heating were freeze-dried, the dry residue was taken up in water (300 ml./kg. of tissue), diluted 2 in 5 with tungstic acid reagent and centrifuged. The foamy yellow extract was purified on Amberlite IR-120(H), the conditions being similar to those for small quantities of tissue; dimensions and rate of the column were adjusted to the larger samples. The HCl-insoluble residue, removed by gravity filtration, contained approx. 20%, and the final extract for chromatography approx. 50%, of the material reacting with the Sakaguchi reagent added to the resin. These extracts were further purified by paper chromatography. The extract was streaked (50 μl./cm.) across Whatman no. 3MM paper, developed in butanol–acetic acid–water, and the bases were eluted; positions were shown by markers of known bases (see Results section), since the extract concentration was too low for clear spots. The eluates were concentrated either by freeze-drying or on the film evaporator.

RESULTS

Concentration of total monosubstituted guanidines

Concentration in different extracts. Table 3 summarizes the results of Sakaguchi assay on various extracts of adult-rat brain. Trichloroacetic acid and tungstic acid extracts have essentially the same concentration (P value of the difference > 0·01). Both methods are reproducible and give good recoveries: in trichloroacetic acid, arginine recovery was 99 ± 4% (S.D.; four determinations) and

Table 3. *Concentration of monosubstituted guanidines in rat-brain extracts*

The cerebrum was removed at room temperature and ground in deproteinizing agent within 3 min. of death (see text). Assay was by the method of Dubnoff. Values are expressed as μmoles of arginine/g. wet wt. (assuming 80% of wet wt. is water), followed by mean deviation and the number of animals examined. Duplicate determinations were made on extracts from each animal.

Deproteinizing agent	Concentration (μmole/g. of fresh tissue)
Trichloroacetic acid	0·21 ± 0·03 (4)
Tungstic acid	0·18 ± 0·02 (4)
Metaphosphoric acid	0·33 ± 0·02 (2)
Somogyi reagents	0·11 ± 0·02 (2)
Heat	0·7 ± 0·1 (4)

γ-guanidobutyric acid recovery was 95 ± 7% (S.D.; four determinations): in tungstic acid, γ-guanidobutyric acid recovery was 101 ± 6% (S.D.; four determinations). Metaphosphoric acid extracts give a significantly higher level, possibly because they contain proteins or peptides which give a Sakaguchi reaction.

Extracts which were deproteinized by heating showed a concentration of guanidine three to five times that with trichloroacetic acid. The increase could be caused by breakdown of a chemical precursor, by release of free arginine from proteins, or by proteins or peptides left in solution under these conditions. Chemical breakdown could not give rise to significant amounts of non-endogenous monosubstituted guanidines; all bases identified were also found in tungstic acid extracts. Even the active brain proteinases should be rapidly inactivated at 95° (Richter, 1959; McIlwain, 1959). The following evidence supports the third interpretation: (1) The extracts contain much surface-active material. (2) Although biuret assay (Layne, 1957) was negative, implying the presence of less than 2·5 mg. of protein/g. of tissue in the solution, Folin–Ciocalteu assay for aromatic amino acids (Layne, 1957) showed 3·9 mg. of protein/g. of tissue left in solution. (3) Tungstic acid precipitated all (about 0·3 μmole of guanidine/g. of tissue; approx. 2 mg. of protein/g. of tissue), and trichloroacetic acid precipitated half of the excess of material reacting with the Sakaguchi reagents.

Concentrations in different tissues. Table 4 summarizes the results of Sakaguchi assay on trichloroacetic acid extracts of brains derived from different sources. No difference was noted, in adult rats and guinea pigs, between extracts of cerebral hemispheres and those of combined cerebral hemispheres and cerebellum. Adult rat and adult guinea-pig brains contain essentially the same concentration of monosubstituted guanidines ($P > 0·01$). The concentration in ox brain is higher and more variable (cf. standard deviations): the differences could arise from proteolysis or similar breakdown during the rather more drastic pretreatment of the ox tissue (see Methods section). The difference in concentration between young rat brain frozen *in situ* and that obtained normally, and between young and adult rats, are both barely significant ($0·01 < P < 0·001$). In these studies, only one batch of young (newly weaned) animals was used.

Table 4. *Concentrations of monosubstituted guanidines in brains from various sources*

Extraction was with trichloroacetic acid; assay was by the method of Dubnoff (see text). Concentrations are expressed as μmole of arginine/g. wet wt. of tissue (assuming 80% of wet wt. is water); the values are followed by S.D. and the numbers of animals examined. Duplicate determinations were made on samples from each animal.

Tissue	Source	Concentration (μmole/g. of fresh tissue)
Cerebral hemispheres or combined cerebral hemispheres and cerebellum	Adult guinea pig	0·24 ± 0·04 (11)
	Adult rat	0·21 ± 0·04 (13)
Cerebral hemispheres	Ox	0·30 ± 0·10 (4)
Cerebral hemispheres	Young rat*	0·29 ± 0·05 (11)
Cerebral hemispheres, frozen *in situ*	Young rat*	0·21 ± 0·04 (7)

* 2-week-old newly weaned animals, weighing about 100 g.

Identification of monosubstituted guanidines

Paper chromatography of the alkaline constituents, concentrated by column chromatography from rat brains, in butanol–acetic acid–water (three experiments, with six animals each), and elution and rechromatography in butanol–pyridine–water (one experiment), both revealed four materials, which moved with arginine, glycocyamine, taurocyamine and γ-guanidobutyric acid respectively. The extracts were too impure to give good two-dimensional chromatograms; even in one-dimensional chromatography, it was necessary to add approx. 2 mg. of total material to the paper to obtain spots bright enough and small enough (about 1·5 cm. diam.) to give a clear colour above background (see Smith, 1958). From the minimum quantity needed to reveal a particular base (see Methods section), the concentrations of the individual bases in the tissue can be roughly calculated, in μmole/g., as: arginine, 0·1; γ-guanidobutyric acid, 0·05; glycocyamine, 0·03; taurocyamine, 0·03.

With extracts of ox brain, two-dimensional chromatography (0·1–0·15 mg.) showed four spots, moving with the known bases (two experiments, each on two ox brains). Eluates obtained from Whatman no 3 MM paper (see Methods section) showed materials which moved with the known bases in the five solvents (see Methods section).

The same four bases were found in ox-brain preparations deproteinized by heating, and purified by solvent extraction and paper chromatography; the procedure was relatively unreproducible. Preparations by other methods, including alkaline silver precipitation (B. M. Bollard & H. McIlwain, unpublished work), electrolytic desalting and Somogyi (1945) precipitating gave no evidence for any other naturally occurring monosubstituted guanidines.

DISCUSSION

Presence of monosubstituted guanidines

Total concentration. The concentration of the monosubstituted guanidines occurring in brain, 0·2–0·3 μmole/g., is markedly smaller than the

8–10 μmoles/g. of total creatine (disubstituted guanidines) (Olsen & Klein, 1947). Most of these investigations have been carried out with tissue removed from animals at room temperature, but it has been shown that tissues frozen *in situ* do contain monosubstituted guanidines.

Arginine. Of the individual guanidines, arginine constitutes nearly half of the total. The present value of 0·1 μmole/g. of rat brain is similar to the 0·12 μmole/g. of rat brain of 'microbiologically available' arginine (Schurr, Thompson, Henderson, Williams & Elvehjem, 1950) and to the 0·08 μmole of free arginine/g. of rat brain (Tallan, Moore & Stein, 1954; Clouet, Gaitonde & Richter, 1957). Paper-chromatographic identification of arginine as one of the free amino acids of brain was reported by Irrevere & Evans (1959) and by Clouet *et al.* (1957), but not by Walker (1952) nor by Awapara, Landau & Fuerst (1957).

γ-Guanidobutyric acid. This was reported in cattle brain (Irrevere, Evans, Hayden & Silber, 1957) and, while these investigations were in progress, was isolated from calf brain by Irrevere & Evans (1959) who found 0·03 μmole/g. of calf brain. We found approximately 0·05 μmole/g. of rat brain.

Glycocyamine. This was identified as a cerebral constituent by Borsook & Dubnoff (1941) and by Clouet *et al.* (1957). Our value for rat brain (0·03 μmole/g.) is significantly lower than that reported by Borsook & Dubnoff (0·15–0·3 μmole/g.) and by Clouet *et al.* (0·1 μmole/g.). The earlier values are based on column purifications which may not have distinguished glycocyamine from γ-guanidobutyric acid and taurocyamine.

Taurocyamine. This has been identified in brain tissue in the present study. It is a constituent of Crustacea (Van Thoai & Robin, 1954) and mammalian urine (Van Thoai, Roche & Olumucki, 1954).

Other monosubstituted guanidines. In extracts prepared by several radically different procedures, only the four bases discussed above were identified. The existence of another simple, monosubstituted guanidine in brain, in concentrations greater than 0·03 μmole/g., seems unlikely.

Function of the guanidines

All of the monosubstituted guanidines identified are ω-guanido acids, although the isolation procedures would not be expected to select these amphoteric guanidines. ω-Guanido acids have been shown to have several effects on neural preparations. They act on the inhibitory synapses in isolated crustacean nerve (Edwards & Kuffler, 1959), and they are among the most active of a series of compounds affecting electric activity of the cerebral cortex (Purpura, Girado, Smith, Callan & Grundfest, 1959). Monosubstituted guanidines inhibit the Pasteur effect in brain slices (Dickens, 1939; McIlwain, 1950). After intracisternal injection of radioactive amino acids into rabbits, activity has been found in Krebs urea-cycle intermediates in the brain (Sporn, Dingman, Defalco & Davies, 1959*a*, *b*; Sporn, Dingman & Defalco, 1959). Rat-brain homogenates contain a transamidinase, synthesizing guanidines from analogous amines (Pisano & Udenfriend, 1958). Preliminary experiments in this laboratory have suggested the presence of an acid-labile, bound form of guanidines in cerebral tissue (H. McIlwain & J. P. Blass, unpublished observations).

SUMMARY

1. Methods of extracting the monosubstituted (Sakaguchi-positive) guanidines of brain, and of their chromatographic characterization, are described.

2. The occurrence of free arginine, glycocyamine and γ-guanidobutyric acid is confirmed, and evidence for the presence of taurocyamine is presented.

3. Concentrations of these compounds are approximately 0·03–0·1 μmole/g. of tissue, which corresponds to about 1 % of the free creatine of the brain.

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REFERENCES

Aves, E. K. (1959). *J. Inst. Sci. Tech.* **5**, no. 4, 6.
Awapara, J., Landau, A. J. & Fuerst, R. (1957). *Biochem. biophys. Acta*, **5**, 457.
Borsook, H. & Dubnoff, J. W. (1941). *J. biol. Chem.* **138**, 389.
Caraway, W. T. (1958). *Chem. Anal.* **47**, 44.
Clouet, D. H., Gaitonde, M. K. & Richter, D. (1957). *J. Neurochem.* **1**, 228.
Dickens, F. (1939). *Biochem. J.* **33**, 2017.
Dubnoff, J. W. (1941). *J. biol. Chem.* **141**, 711.
Dubnoff, J. W. (1957). In *Methods in Enzymology*, vol. 3, p. 635. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Edwards, C. & Kuffler, S. W. (1959). *J. Neurochem.* **4**, 191.
Engel, R. (1875). *Ber. dtsch. chem. Ges.* **8**, 1597.
Engeland, R. & Kutscher, F. (1910). *Ber. dtsch. chem. Ges.* **43**, 2882.
Hunter, G. (1957). *J. clin. Path.* **10**, 161.
Irrevere, F. & Evans, R. L. (1959). *J. biol. Chem.* **234**, 1438.
Irrevere, F., Evans, R. L., Hayden, A. R. & Silber, R. (1957). *Nature, Lond.*, **180**, 704.
King, H. (1930). *J. chem. Soc.* p. 2374.

Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 448. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
McIlwain, H. (1950). *Biochem. J.* **46**, 612.
McIlwain, H. (1959). *Biochemistry and the Central Nervous System*, 2nd ed., p. 183. London: J. and A. Churchill Ltd.
McIlwain, H., Buchel, L. & Cheshire, J. D. (1951). *Biochem. J.* **48**, 12.
Morgue, M. (1948). *Bull. Soc. chim. Fr.* p. 181.
Morrison, J. F., Ennor, O. H. & Griffiths, D. E. (1958). *Biochem. J.* **68**, 447.
Olsen, N. S. & Klein, J. R. (1947). *J. biol. Chem.* **167**, 739.
Pisano, J. J. & Udenfriend, S. (1958). *Fed. Proc.* **17**, 403.
Purpura, D. P., Girado, M., Smith, T. G., Callan, D. A. & Grundfest, H. (1959). *J. Neurochem.* **3**, 238.
Richter, D. (1959). *Proc. 4th int. Congr. Biochem., Vienna*, **3**, 173.
Richter, D. & Dawson, R. M. C. (1948). *Amer. J. Physiol.* **154**, 73.
Robin, Y. (1954). *Répartition et Métabolisme des Guanidines d'Origine Animale*, p. 27. Lons-le-Saunier: Declume.
Schurr, P. E., Thompson, H. T., Henderson, L. M., Williams, J. N., jun. & Elvehjem, C. A. (1950). *J. biol. Chem.* **182**, 39.
Schütte, E. (1943). *Hoppe-Seyl. Z.* **279**, 52.
Smith, I. (1958). *Chromatographic Techniques*, p. 149. London: William Heinemann (Medical Books) Ltd.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.
Sporn, M. B., Dingman, W. & Defalco, A. (1959). *J. Neurochem.* **4**, 141.
Sporn, M. B., Dingman, W., Defalco, A. & Davies, R. K. (1959*a*). *Nature, Lond.*, **183**, 1520.
Sporn, M. B., Dingman, W., Defalco, A. & Davies, R. K. (1959*b*). *J. Neurochem.* **5**, 62.
Tallan, H. H., Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 927.
Van Pilsum, J. F., Martin, R. P., Kito, E. & Hess, J. (1957). *J. biol. Chem.* **222**, 225.
Van Thoai, N. & Robin, Y. (1954). *Biochem. biophys. Acta*, **13**, 533.
Van Thoai, N., Roche, J. & Olumucki, A. (1954). *Biochem. biophys. Acta*, **14**, 448.
Walker, D. M. (1952). *Biochem. J.* **52**, 679.

Biochem. J. (1960) **77**, 489

The Incorporation of Radioactive Amino Acids into Proteins of the Microsome Fraction of Guinea-Pig Liver at very Short Time Intervals after Administration

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The question whether the process of incorporation of labelled amino acids into trichloroacetic acid-precipitable protein, which takes place in cell-free systems, represents part or all of the process resulting in the synthesis of proteins in the corresponding intact cell is an important problem in the interpretation of the results of many investigations into the mechanism of protein biosynthesis. Simkin & Work (1957*a*, *b*) attempted to obtain information on this question with respect to the microsome fraction of guinea-pig liver. In that work the microsome fraction was labelled with [^{14}C]amino acids by allowing incorporation to take place either in the intact guinea pig or in a cell-free system of the type described by Zamecnik & Keller (1954) which contains microsome and cell-sap fractions. The microsome fraction was then isolated, and separated into a series of distinct subfractions by a procedure involving successive extraction with solutions of varied ionic strength and pH. The radioactivity of the protein of the subfractions was determined. When the results of labelling under the two sets of conditions were compared, the pattern of labelling found in the cell-free system appeared to be different from that resulting from incorporation in the intact cell. The difference in behaviour was particularly noticeable in two ribonucleic acid-rich subfractions, *B* and *C*. The reasons for this difference in the labelling pattern were not clear. The shortest period of exposure to radioactive amino acids *in vivo* in this work was 2 min., and, as Simkin (1959) has pointed out, the possibility could not therefore be excluded that the pattern of labelling found in the cell-free system resembled that occurring in the intact liver cell as a result of periods of incorporation less than this. The results of analogous investigations carried out by Sachs (1958) lent support to this view. In order to resolve this question, the labelling of the microsome subfractions of guinea-pig liver occurring *in vivo* has been re-examined, very brief periods of exposure to [^{14}C]amino acids now being employed. The results obtained support the suggestion made above: there is a similarity in the pattern of labelling occurring in

the cell-free system to that found as a result of a period of incorporation of about 30 sec. in the intact liver cell. Some reinterpretation of our earlier work is therefore necessary.

EXPERIMENTAL

Animals. Guinea pigs were purchased from A. J. Tuck and Son, Rayleigh, Essex. The animals, of 630–750 g. body wt., were starved overnight before use.

Radioactive amino acids. A mixture of uniformly labelled L-[^{14}C]amino acids was prepared by acid hydrolysis of *Chlorella* [^{14}C]protein and purified by passage through a column of Zeo-Karb 225 as described by Simkin (1958). The solution used for injection contained amino acids (100 μc/ml.; 5 μmoles of N/ml.). in 0·9% sodium chloride.

Administration of radioactive amino acids and removal of liver. The animals were anaesthetized by combined intraperitoneal injection of sodium pentobarbitone (37–47 mg./kg. body wt., in the form of 0·40–0·55 ml. of oven Veterinary Nembutal, Abbott Laboratories, London) and inhalation of ether. The abdomen was opened and ligatures were loosely tied around the posterior vena cava anterior to the liver and around the portal vein. The purpose of the ligatures was to interrupt a major part of the supply of blood to the liver after the appropriate period of incorporation had been allowed to take place, in an effort to slow down any incorporation of amino acids which might occur during excision of the liver (cf. Loftfield & Eigner, 1958). In addition, the ligatures facilitated rapid removal of the liver. The [^{14}C]amino acid solution was injected into the portal vein about 2 cm. from the liver; the quantities of isotope administered were 80, 75 and 63 μc/kg. body wt. in Expts. 1, 2 and 3, respectively. After the appropriate time interval had elapsed, the ligatures were pulled tight, and the liver removed, immersed in a bath of ice-cold 0·25 M-sucrose, and immediately cut into pieces with scissors. The whole operation was carried out as rapidly as possible, and efforts were made to keep the animals warm.

Isolation and fractionation of microsome material. The procedure used followed closely that adopted in earlier work. In brief, the livers were fractionated by differential centrifuging as described by Simkin & Work (1957*a*). The microsome material so obtained was washed and fractionated by the technique employed for material which had been labelled in a cell-free system (Simkin & Work, 1957*b*); this method represents a slight modification of the original technique (Simkin & Work, 1957*a*). The fractionations were checked by determining the ultraviolet-absorption spectra of the microsome subfractions (Simkin & Work, 1957*a*).

Isolation of protein for measurement of radioactivity and determination of radioactivity. The methods employed were those detailed by Simkin & Work (1957*a*). In summary, after addition of a mixture of non-radioactive amino acids, protein was precipitated from samples by addition of trichloroacetic acid. The precipitates were dissolved in alkali in the presence of non-radioactive amino acids and protein again precipitated by the addition of trichloroacetic acid; the precipitates were then washed in order to remove nucleic acid and lipid. Radioactivity was determined with a thin end-window counter. With few exceptions, the standard deviation of the net count rate was not greater than $\pm 5\%$, and more often was ± 1 or 2%. With a few samples of very low specific activity, for practical reasons the standard deviation was greater, the highest value being $\pm 13\%$ (subfraction *A*, Expt. 2). Under the conditions of counting used, 1 cm.^{2} of methacrylate resin of 10^{-3} μc/mg. gives 1000 counts/min. at infinite thickness.

RESULTS

The results are presented of three experiments on the incorporation *in vivo* of [^{14}C]amino acids (*Chlorella* protein hydrolysate) into protein of subfractions of the microsome material of guinea-pig liver. In two experiments, (1 and 2), incorporation was allowed to take place for periods of time, namely 0·4 and 0·75 min., which were shorter than any used in the earlier investigation of Simkin & Work (1957*a*). The other experiment (3), was designed to determine whether the surgical procedure or anaesthetic used in the present work affected the pattern of labelling obtained, and in this instance a period of incorporation was employed, viz. 3·5 min., which lay between the two shortest time intervals which had been used in the earlier study in order to facilitate comparison with the results obtained in that work. The time intervals stated represent the time elapsing from the beginning of the injection of [^{14}C]amino acids into the portal vein to the time when the ligatures around the posterior vena cava and portal vein (see Experimental section) were pulled tight. The injection of the amino acid solution was completed within 6, 4 and 2 sec. in Expts.1 2 and 3, respectively. After the interruption of the blood supply to the liver, a period of 9–10 sec. was required for excision of the liver and transfer to ice-cold 0·25 M-sucrose.

The results (Fig. 1) are given, as in earlier papers (Simkin & Work, 1957*a*, *b*), in terms of the ratio of the specific radioactivity of the protein of a microsome subfraction to the specific radioactivity of the protein of the whole microsome fraction at the corresponding time interval. This method of expression facilitates comparison of data obtained in different experiments. The actual specific radioactivities of the protein of the whole microsome fractions in Expts. 1, 2, and 3 were 43, 182 and 706 counts/min./cm.^{2} at infinite thickness, respectively. Fig. 1 shows that at short time intervals after injection of [^{14}C]amino acids, the protein of subfraction *A* (a fraction extracted by 0·14 M-NaCl, and containing mainly protein) possessed only a very low level of radioactivity when compared with the protein of the microsome fraction as a whole. After the shortest period of incorporation (about 0·4 min.), the protein of the ribonucleic acid (RNA)-rich subfraction *B*, isolated by extraction with M-NaCl, had a specific activity over twice as great as that possessed by the whole microsome protein. As the period of exposure to isotope was increased, however, the specific activity relative to that of the whole microsome protein decreased markedly. In

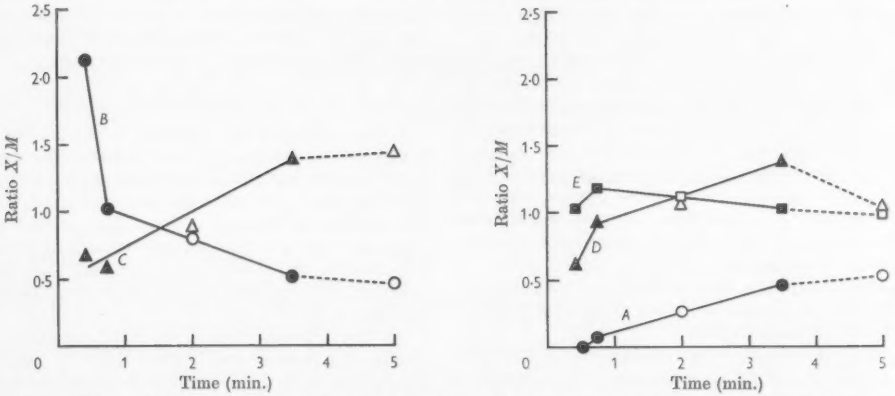

Fig. 1. Change with time of the radioactivity of proteins of subfractions (*A–E*) of microsome material from guinea-pig liver after injection of [^{14}C]amino acids. Radioactivity is expressed as the ratio of the specific activity of the protein of the subfraction (*X*) to that of the corresponding whole microsome protein (*M*). The values represented by the open symbols are those obtained in previous work (Simkin & Work, 1957*a*) in which unanaesthetized animals, not subjected to surgical disturbance, were used.

contrast, after either 0·4 or 0·75 min. incorporation, the specific activity of the protein of subfraction *C*, another RNA-containing fraction, extracted by 0·1 M-bicarbonate–carbonate buffer, pH 9·0, was about two-thirds that of the corresponding whole microsome protein, but the relative activity of the protein of *C* increased appreciably as a result of longer periods of incorporation. The proteins of the lipid-containing subfractions *D* and *E*, separated by means of their differing solubility in 0·01 N-NaOH, showed a slightly different behaviour to one another. The protein of *D* had at first a lower activity than that of the whole microsome protein, but later possessed an activity equal to, or slightly greater than, the whole. The protein of *E* possessed a rather similar specific activity to the whole microsome protein at each time studied.

The data presented in Fig. 1 indicate that there is excellent agreement between the results of Expt. 3 of the present investigation (3·5 min. incorporation) and the values which might be expected on the basis of earlier work for the incorporation pattern resulting from such a period of labelling. It therefore seems very probable that the surgical procedure, anaesthetic, or different route of administration of amino acid used in the present work do not affect the pattern of labelling obtained to any appreciable extent.

DISCUSSION

The results of the present investigation, when combined with the data obtained in earlier work (Simkin & Work, 1957*a*), indicate that the pattern of incorporation of amino acids into the protein of subfractions of guinea-pig-liver microsome material is constantly changing as the time interval after administration of labelled amino acid increases. With subfractions *A*, *B*, *C* and probably *D*, there is an increase in the value of the ratio of specific activity of subfraction protein to specific activity of corresponding whole microsome protein, followed by a decrease in the magnitude of the ratio. The proteins of the subfractions of high RNA content, *B* and *C*, exhibit this kind of behaviour to the most marked extent: in both instances, the maximum value for the ratio exceeds 2·0, the maximum for *B* being 0·5 min. or less after administration of amino acid and that for *C* about 30 min. later. The reasons underlying the complexity of the labelling of the microsome proteins remain obscure. The very rapid labelling of the protein of subfraction *B* was not detected in our earlier work since the shortest time period of incorporation investigated was 2 min. On the basis of the results then obtained, it was suggested that material contained in subfraction *C* might be of importance in the process of amino acid incorporation since the protein of *C* attained a particularly high level of radioactivity. There appeared to be an analogy with the behaviour of RNA-containing subfractions of other kinds isolated from rat-liver microsome material by a number of investigators (for some references, see Simkin & Work, 1957*a*), with the exception of the fact that the protein of other ribonucleoprotein fractions gained and lost radioactive amino acids at a much more rapid rate than did the protein of subfraction *C*. Thus, when Littlefield, Keller, Gross & Zamecnik (1955) studied the incorporation of [^{14}C]amino acids into the ribosomes of rat-liver microsome material, they found that the maximum specific

activity of the ribosome protein was reached 3 min. or less after the administration of amino acid. Much subsequent work has supported the suggestion of Littlefield *et al.* (1955) that the ribosomes are the primary site of polymerization of amino acids in the cytoplasmic material comprising the microsome fraction. It is now clear that it is the protein of the other RNA-rich subfraction, *B*, that behaves in an analogous way to that of ribosome protein and may therefore, at least in part, be derived from it. It is perhaps significant that the protein of subfraction *B* has a high content of arginine (Simkin & Work, 1957*b*) as, characteristically, has the ribosome protein of rat liver (Crampton & Petermann, 1959).

When the results of the application of the fractionation procedure to guinea-pig-liver microsome material labelled in a cell-free system (Simkin & Work, 1957*b*) are compared with those obtained in the present investigation, there is in fact a substantial degree of similarity between the pattern produced in the cell-free system as a result of about 15 min. of incubation and that which results from about 0·4 min. of exposure to labelled amino acid *in vivo*. The results are particularly striking for subfractions *B* and *C*: in either case, the protein of *B* has a specific activity over twice as great as that of the whole microsome protein, whereas the protein of *C* has a specific activity about 60–70 % of that of the whole protein. If incorporation is prolonged beyond this stage, the activity, relative to the total protein, of the protein of *B* decreases whereas that of *C* increases. With the cell-free system, however, incorporation into microsome protein as a whole ceases before this second stage is far advanced. There is also a general similarity in the behaviour of the proteins of subfractions *A*, *D* and *E* in the cell-free system and in the early stages of incorporation *in vivo*. It thus seems possible that the pattern of labelling in the cell-free system might result from the operation of a mechanism of amino acid incorporation which is possessed by the intact liver cell. On this view, the mechanism would operate in the cell-free system in an essentially similar manner to normal but at a much lower rate, i.e. about one-twentyfifth to one-fiftieth of that in the intact cell. The results of Sachs (1958), who used a different fractionation procedure to examine rat-liver microsome material labelled *in vivo* or in a cell-free system, are, where comparison can be made, in general agreement with those considered here. The findings of a number of recent investigations (e.g. Campbell, Greengard & Kernot, 1960) into protein synthesis, in cell-free systems of the type considered above, are consistent with the view that the process of incorporation in such systems represents, at least in part, the process resulting in the synthesis of protein in the intact cell. It is only by the study of the synthesis of specific proteins in cell-free systems that any definitive answer to the question of the validity of the use of such systems can be obtained.

SUMMARY

1. In extension of previous work (Simkin & Work, 1957*a*), guinea pigs were killed at short time intervals after the intraportal injection of a mixture of [^{14}C]amino acids (*Chlorella* protein hydrolysate). The microsome fraction of liver was isolated, and separated into a number of subfractions of distinct composition by a procedure involving successive extraction with solutions of varied ionic strength and pH.

2. The patterns of labelling of the protein of the microsome subfractions found after periods of incorporation of approx. 0·4 or 0·75 min. were different from those resulting from longer periods of incorporation and which have been reported earlier. In particular, the protein of the ribonucleic acid-rich subfraction *B* had a specific activity over twice that of the microsome proteins as a whole after 0·4 min. incorporation; this high relative value fell markedly, however, as the period of exposure to labelled amino acid was increased.

3. Earlier studies (Simkin & Work, 1957*a*, *b*) of the pattern of labelling of the protein of microsome subfractions, resulting from incorporation either *in vivo* or in a cell-free system, have been reinterpreted in the light of the present findings. The incorporation of amino acids into microsome protein proceeded for only about 20 min. in the cell-free system used, and the pattern of labelling found at that time is seen to be essentially similar to that produced by about 0·5 min. of exposure to labelled amino acid *in vivo*. The implications of this finding are discussed.

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REFERENCES

Campbell, P. N., Greengard, O. & Kernot, B. A. (1960). *Biochem. J.* **74**, 107.
Crampton, C. F. & Petermann, M. L. (1959). *J. biol. Chem.* **234**, 2642.
Littlefield, J. W., Keller, E. B., Gross, J. & Zamecnik, P. C. (1955). *J. biol. Chem.* **217**, 111.
Loftfield, R. B. & Eigner, E. A. (1958). *J. biol. Chem.* **231**, 925.
Sachs, H. (1958). *J. biol. Chem.* **233**, 643.
Simkin, J. L. (1958). *Biochem. J.* **70**, 305.
Simkin, J. L. (1959). *Annu. Rev. Biochem.* **28**, 145.
Simkin, J. L. & Work, T. S. (1957*a*). *Biochem. J.* **65**, 307.
Simkin, J. L. & Work, T. S. (1957*b*). *Biochem. J.* **67**, 617.
Zamecnik, P. C. & Keller, E. B. (1954). *J. biol. Chem.* **209**, 337.

Studies in Detoxication

84. THE METABOLISM OF [^{14}C]ANILINE IN THE RABBIT AND OTHER ANIMALS*

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The fate of aniline in the rabbit was investigated by Smith & Williams (1949), who showed that glucuronides and ethereal sulphates of *o*- and *p*-aminophenol, and possibly 4-aminoresorcinol, were metabolites accounting for 40–45% of the aniline fed; but the main metabolite, more than 50% of the dose, was considered to be a labile glucuronide of aniline itself. This labile *N*-glucuronide was also thought to be the principal metabolite of aniline by Ishidate, Hogiya, Hashimoto & Takitani (1958); and another conjugate of aniline, phenylsulphamic acid, was detected in the urine of rabbits dosed with aniline (Boyland, Manson & Orr, 1957). The metabolism of aniline by liver slices from rat, rabbit, dog and cat has yielded *o*- and *p*-aminophenol and 4-aminoresorcinol (Sato, Suzuki, Fukuyama & Yoshikawa, 1955); rat-liver and cat-liver microsomes in the presence of reduced triphosphopyridine nucleotide and oxygen gave *o*- and *p*-aminophenol (Booth & Boyland, 1957; H. S. Posner, unpublished results), and rabbit-liver microsomes under the same conditions gave only *p*-aminophenol (Mitoma, Posner, Reitz & Udenfriend, 1956). The hydroxylation of aniline to *p*-aminophenol has been effected by ascorbic acid in the presence of ferrous iron, ethylenediaminetetra-acetic acid and oxygen (Brodie, Axelrod, Shore & Udenfriend, 1954), and it appears likely that ascorbic acid is involved in the hydroxylation of aniline *in vivo* (Axelrod, Udenfriend & Brodie, 1954; Volkova, 1958). The present work with [^{14}C]aniline has shown that the principal fate of aniline in mammals is hydroxylation to *o*- and *p*-aminophenol, with the *N*-glucuronide of aniline as only a minor metabolite.

The use of [^{14}C]aniline has made possible the accurate estimation in urine of the isomeric aminophenols, aniline *N*-glucuronide, phenylsulphamic acid and acetanilide, and of the amounts exhaled in the breath and retained in the faeces and tissues. In this way some 80–100% of a single dose of aniline has been accounted for. In addition, the principal metabolites have been estimated in several animal species and the proportion of *o*- to *p*-aminophenol excreted by different animals has been shown to vary widely.

* Part 83: Baldwin, Robinson & Williams (1960).

MATERIALS AND METHODS

Melting points are corrected.

Preparation of [^{14}C]*aniline.* [^{14}C]Aniline randomly labelled with ^{14}C in one carbon atom was prepared from [^{14}C]nitrobenzene by catalytic transfer-hydrogenation with *cyclo*hexane and palladium black according to Braude, Linstead & Wooldridge (1954) (yield 91%, b.p. 184°).

Aniline N-glucuronide. Salts of aniline-*N*-glucosiduronic acid have been previously described (Thierfelder, 1889; Bergmann & Wolff, 1923; Ishidate *et al.* 1958), but unequivocal characterization of these unstable compounds was first established by Heyns & Baltes (1958), who described a sodium salt, $C_{12}H_{14}O_6NNa,\frac{1}{2}C_6H_7N$, m.p. 201–203° (decomp.), and a potassium salt, $C_{12}H_{14}O_6NK,C_6H_7N,H_2O$, m.p. 175° (decomp.). An hydrated ammonium salt, m.p. 130° (decomp.), $[\alpha]_D^{24}$ −55° → −15° (*c*, 2·3 in water), was described but not adequately characterized by Ishidate *et al.* (1958). The ammonium salt used in experiments described in this paper was prepared as follows: to a solution of 5 g. of glucurone in 15 ml. of water was added 1·9 ml. of aq. NH_3 soln. (sp.gr. 0·88) followed by 5 ml. of aniline in 10 ml. of ethanol. After mixing, the solution was left at room temperature until a precipitate began to form. The mixture was then kept at 0° for 8 hr. and the precipitate was filtered off and washed with a little cold ethanol. Recrystallization from aq. 50% (v/v) ethanol gave *ammonium aniline-N-glucosiduronate*, containing 1 mol.prop. of aniline of crystallization, as colourless needles, m.p. 122° (decomp.); $[\alpha]_D^{22}$ −77° → −17°±1° (*c*, 2·0 in water) (Found: C, 54·7; H, 6·65; N, 10·4; aniline, 47·3. Calc. for $C_{12}H_{18}O_6N_2,C_6H_7N$, H_2O: C, 54·4; H, 6·85; N, 10·6; aniline, 46·9%). The total aniline content was estimated by refluxing 0·05 g. of the salt with 10 ml. of 2N-HCl for 1 hr., steam-distilling the solution after adjusting to pH 10 with 5N-NaOH, and determining the difference in light absorption of the steam-distillate at 280 mμ in 0·1N-HCl and 0·1N-NaOH (aniline in 0·1N-NaOH showed light-absorption at 280 mμ, ϵ 1300; in 0·1N-HCl at 280 mμ, ϵ 0). The ammonium salt is readily soluble in cold water, less soluble in ethanol and insoluble in ether and ethyl acetate. It slowly reduces Benedict's reagent but reduces more readily after preliminary treatment with N-HCl. It gives the naphtharesorcinol reaction for glucuronic acid and shows the colour reactions characteristic of free aniline. The structure of the ammonium salt is similar to that of the potassium salt and not the sodium salt, as would be expected, since the radius of the ammonium ion, 1·48 Å, is closer to that of potassium (1·33 Å) than that of sodium (0·95 Å).

Other compounds. 3-Aminocatechol–HCl and 7-hydroxy-2-phenylbenzoxazole, m.p. 192° (Henrich, 1921); 4-aminocatechol–HBr (Jacobs, Heidelberger & Rolf, 1919) and

4-benzamidocatechol, m.p. 169° (Kehrmann & Hoehn, 1925); 2-aminoresorcinol–HCl and 2-benzamidoresorcinol, m.p. 190° (Kauffmann & dePay, 1906); 4-aminoresorcinol–HCl and 4-benzamidoresorcinol dibenzoate, m.p. 172° (Henrich & Wagner, 1902); 5-aminoresorcinol–HCl and 5-acetamidoresorcinol diacetate, m.p. 121° (Pollack, 1893); aminoquinol–HCl (Henrich, 1921) and acetamidoquinol, m.p. 100° (Kehrmann & Bahatrian, 1898); *p*-aminothiophenol, m.p. 46° (Hinsberg, 1906) and 4:4′-diaminodiphenyl disulphide, m.p. 81° (Hodgson & Dix, 1914); *p*-aminophenylglucuronide, m.p. 215° (Williams, 1943) and *p*-acetamidophenylglucuronide, m.p. 193° (Smith & Williams, 1948); and barium phenylsulphamate (Traube, 1890) were also prepared. Other compounds required for isotope-dilution experiments were purchased or prepared by standard methods and purified to constant m.p.

Animals. The [^{14}C]aniline contained about 100 μc/ml. and was administered, alone or suitably diluted with inactive aniline (A.R.), b.p. 184–185°, by stomach tube to gerbils, guinea pigs, hamsters, rabbits, rats, mice, ferrets, cats, dogs and hens. The mice, rats, gerbils, rabbits and hamsters were maintained on Diet 41 B (E. Dixon and Son, Ltd., Ware, Herts.); guinea pigs on diet 18 (E. Dixon and Son, Ltd.) and cabbage; ferrets and dogs on milk together with raw beef and wholemeal bread in equal amounts; cats on milk and Kit-e-Kat; and hens on SPBRA 'Battery intensive mash'. Both food and water were available without restriction to all animals. All animals were female except where stated to the contrary.

The rabbits, after dosing, were placed in a metabolism chamber and the expired air was collected and analysed for unchanged aniline and CO_2 as described previously (Parke, 1956). They were removed from the chamber 3 or 4 days after dosing, killed and their tissues were analysed for radioactivity. The daily volume of urine was approximately 100 ml., and portions of 1–10 ml./day, according to the doses of ^{14}C, were taken for the isotope-dilution procedures. The other animals were placed in open metabolism cages after dosing and their urine was collected and analysed daily.

Measurement of radioactivity. Measurements were carried out on solid and liquid samples of infinite thickness on nickel-plated iron planchets (General Electric Co., Ltd.) with an end-window counter tube, the background of which was 7–9 counts/min. The specific activities were determined by comparison with a stable polymer reference (The Radiochemical Centre, Amersham, Bucks.), which was standardized against [^{14}C]acetanilide, prepared from the labelled aniline. A sample of 1·75 cm.^{2} containing 1 μc of ^{14}C/g. of substance gave approximately 1080 counts/min. The variation in disk size and preparation of sample gave an error of $\pm 5\%$.

Urines and tissues. The total radioactivity of the urines and tissues were measured at $BaCO_3$ obtained by wet combustion (see Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). In some experiments the urine was counted directly as a liquid and the tissues were counted as homogenates. There were no significant differences between the results from these two methods. The limits of detection of metabolites in the urine were of the order of 0·02% of the dose.

Analytical methods. Glucuronic acid and ethereal sulphate in urine were determined as described by Mead, Smith & Williams (1958).

Estimation of metabolites

Carbon dioxide. Each 4 hr. fraction of the CO_2 absorbed in the NaOH was converted into $BaCO_3$ and the radioactivity measured as described by Calvin *et al.* (1949). Radioactivity was detected in trace amounts in the expired CO_2 only on the second day after dosing and in 3 or 4 days amounted to less than 0·4% of the dose.

Aniline in the expired air. The aniline eliminated in the expired air was estimated spectrophotometrically with a Unicam spectrophotometer SP. 500. The selected u.v.-absorption maximum of aniline in ethanol was 233 mμ, where ϵ was 11 500 (slit width 0·5 mm.). By this means it was shown that the amount of aniline excreted in the expired air was less than 0·02% of the dose/day. Moreover it is unlikely that any other metabolite of aniline was eliminated in the expired air since the absorption spectra of the ethanolic solutions showed no significant maxima, and the radioactivity of the ethanol corresponded to less than 0·05% of the dose/day.

Aniline in the urine. (i) Free aniline was estimated by adding aniline–HCl (250 mg.) to a portion of the urine (5–10 ml.), adjusting the solution to pH 8·0 with $NaHCO_3$ and extracting the aniline with ether (2 × 10 ml.). The ethereal extracts were washed with water and the aniline was extracted into 2N-HCl (10 ml.). The acid extract was made alkaline (pH 10) with 2N-NaOH and steam-distilled, and the steam-distillate treated with acetic anhydride and 2N-NaOH to yield acetanilide. This was filtered off, washed with water and recrystallized from water to constant specific radioactivity (m.p. and mixed m.p. 114°). (ii) Total aniline was estimated by refluxing a portion of the urine (5–10 ml.) with aniline (250 mg.) and 0·5 vol. of conc. HCl for 3 hr. The solution was made alkaline with 2N-NaOH, steam-distilled and the aniline in the distillate was converted into acetanilide and recrystallized to constant specific radioactivity as before.

Aniline conjugates. The urines were examined for aniline-*N*-glucuronide, phenylsulphamic acid, acetanilide, formanilide, succinanilide and succinanilic acid. Acylations other than acetylation were considered since the transfer of formyl groups is well known (Huennekens, Osborn & Whiteley, 1958), and acyl esters of coenzyme A other than acetyl have been demonstrated (see Williams, 1959).

Aniline-N-glucuronide. Aniline glucuronide (1 g.) was added to a portion of the urine (about 5 ml.), an equal volume of ethanol was added and the mixture was heated to dissolve the glucuronide. On cooling, the glucuronide separated and this was repeatedly recrystallized from aq. ethanol to constant specific radioactivity (m.p. 122°, decomp.). The pure aniline glucuronide was then hydrolysed by refluxing with 2N-H_2SO_4 (10 ml.) for 0·5 hr., and the aniline was steam-distilled, after it was made alkaline with 2N-NaOH, and converted into acetanilide (m.p. and mixed m.p. 114°) as described previously.

Phenylsulphamic acid. Barium phenylsulphamate (2 g.) was dissolved in 10 ml. of ice-cold N-HCl, a portion of the urine (5–10 ml.) was then added and the solution was adjusted to pH 8·0 by dropwise addition of ammonia solution (sp.gr. 0·88). The solution was heated, filtered hot and cooled to allow the barium phenylsulphamate to crystallize. The barium salt was filtered off, washed with cold ethanol and recrystallized twice from water, then repeatedly from aq. ethanol (1:1) until the specific radioactivity was constant. The purified barium phenylsulphamate was then

hydrolysed by refluxing in 2N-HCl (10 ml.) for 0·5 hr. and the aniline was steam-distilled and converted into acetanilide (m.p. and mixed m.p. 114°) as previously described.

Anilides. Acetanilide, formanilide, succinanilide or succinanilic acid (250 mg.) dissolved in 10 ml. of hot water was added to a portion of the urine (10 ml.). After 1 hr. the mixture was extracted three times with 20 ml. portions of ether (acetanilide and formanilide) or $CHCl_3$ (succinanilide and succinanilic acid), the extracts were bulked, washed with 2N-HCl and then water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residues were recrystallized to constant specific radioactivity; acetanilide from water (m.p. and mixed m.p. 114°), formanilide from light petroleum (b.p. 60–80°) (m.p. and mixed m.p. 50°), succinanilide from ethanol (m.p. and mixed m.p. 230°) and succinilic acid from ethanol and then from water (m.p. and mixed m.p. 145°).

Aminophenols. *o*- and *p*-Aminophenol were estimated as described by Parke (1956). *m*-Aminophenol (500 mg.) was added to a portion of the urine (10–50 ml.), which was then refluxed with an equal volume of conc. HCl for 3 hr. The solution was cooled, adjusted to pH 8·0 with solid $NaHCO_3$ and extracted three times with an equal volume of ether. The ethereal extracts were bulked, washed with a little water and the *m*-aminophenol was transferred into 20 ml. of 2N-H_2SO_4. The acid solution was again adjusted to pH 8·0 with $NaHCO_3$ and extracted three times with an equal volume of ether; the ethereal extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue of *m*-aminophenol was dissolved in 2 ml. of ethanol and filtered to remove the less soluble *o*- and *p*-aminophenol. The solution was then applied to Whatman no. 1 paper and chromatographed in wet di*iso*propyl ether (see Table 1); the *m*-aminophenol band, R_F 0·60, was cut out and eluted with acetone. The eluate was evaporated to dryness and the residue benzoylated to yield the *ON*-dibenzoyl derivative of *m*-aminophenol, which was recrystallized from ethanol, toluene and carbon tetrachloride to constant specific radioactivity and had m.p. and mixed m.p. 156°.

Acetamidophenols. *o*- or *p*-Acetamidophenol (250 mg.), dissolved in 10 ml. of hot water, was added to 10 ml. of urine. The mixture was continuously extracted with ether for 6 hr. and the ethereal solution washed with N-HCl, N-Na_2CO_3 and water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue of *o*-acetamidophenol, m.p. and mixed m.p. 203°, was recrystallized from ethanol and then methanol to constant specific radioactivity. The residue of *p*-acetamidophenol, m.p. and mixed m.p. 168°, was recrystallized from aq. ethanol to constant specific radioactivity.

p-*Aminophenylglucuronide.* The glucuronide (1 g.) dissolved in 5 ml. of saturated $NaHCO_3$ soln. was added to a portion of urine (5–10 ml.). The mixture was then acidified to pH 4·0 with 2N-HCl and the precipitated *p*-aminophenylglucuronide was filtered off, washed with water, dissolved in saturated $NaHCO_3$ and reprecipitated at pH 4·0. The glucuronide was finally recrystallized from water (charcoal) to constant specific radioactivity and had m.p. and mixed m.p. 215°.

p-*Acetamidophenylglucuronide.* The glucuronide (1 g.) was dissolved in a portion of the urine (5–10 ml.), which was then evaporated to dryness *in vacuo* at room temperature. The residue was dissolved in 10 ml. of 95% ethanol, the solution was filtered, treated with 1 ml. of benzylamine and then diluted with 100 ml. of ethyl acetate. After being left at 0° for 1 hr. the crystalline precipitate of the benzylamine salt of *p*-acetamidophenylglucuronide was filtered off and was recrystallized from 95% ethanol to constant specific radioactivity (m.p. 196°).

Dihydroxyanilines. 3-Aminocatechol–HCl, 4-aminocatechol–HBr, 2-, 4- and 5-aminoresorcinol–HCl and aminoquinol–HCl (500 mg. each) were added separately to portions of the urine (5–10 ml.), which were refluxed for 3 hr. with an equal volume of conc. HCl and then evaporated to dryness *in vacuo*. The residue of 3-aminocatechol–HCl was refluxed with 3 g. of benzoyl chloride for 1 hr., and the product, the benzoyl derivative of 7-hydroxy-2-phenylbenzoxazole, was distilled, the fraction above 300° being collected. This was then refluxed for 1 hr. with 5 ml. of ethanolic 10% KOH, and the ethanol was distilled off. The residue was washed with water and the insoluble 7-hydroxy-2-phenylbenzoxazole was filtered off and recrystallized from ethanol and then benzene to constant specific activity (m.p. and mixed m.p. 192°). The residues of 4-aminocatechol and 2-aminoresorcinol were redissolved in water (2–5 ml.) and treated with 1 equiv. of benzoyl chloride and excess of sodium acetate to give the *N*-benzoyl derivatives, 4-benzamidocatechol, m.p. and mixed m.p. 169°, and 2-benzamidoresorcinol, m.p. and mixed m.p. 190°, which were recrystallized from ethanol and aq. ethanol to constant specific radioactivity. The residue of 4-aminoresorcinol was dissolved in 5 ml. of water and treated with excess of benzoyl chloride and NaOH to give 4-benzamidoresorcinol dibenzoate, which was recrystallized from light petroleum (b.p. 60–80°) (m.p. and mixed m.p. 172°). The residue of 5-aminoresorcinol was refluxed for 2 hr. with 2 ml. of acetic

Table 1. *R_F values and colour reactions on paper of aminophenols*

R_F values are for descending chromatography on Whatman no. 1 paper in di*iso*propyl ether saturated with water, run for 6 hr. The di*iso*propyl ether was washed with a saturated aq. soln. of $KMnO_4$, a saturated aq. soln. of $FeSO_4$, aq. 2N-Na_2CO_3 and then water, and it was dried over anhydrous Na_2SO_4 and redistilled (b.p. 68°). Detecting reagents used were Gibb's reagent (consisting of ethanolic 2% soln. of 2:6-dichloroquinonechloroimide followed by 2N-Na_2CO_3) and diazo reagent consisting of a 1% soln. of $NaNO_2$ in 0·5N-HCl followed by a 1% soln. of dimethyl-α-naphthylamine in ethanol and then 2N-Na_2CO_3.

		Colour reactions		
			Diazo reagent	
	R_F	Gibb's reagent	Acid	Alkaline
o-Aminophenol	0·79	Blue	Yellow	Green
m-Aminophenol	0·60	Violet	Red	Yellow
p-Aminophenol	0·38	Blue-brown	Violet	Green

anhydride and the product of 5-acetamidoresorcinol diacetate (m.p. and mixed m.p. 121°) was recrystallized from benzene and then water (charcoal). The residue of aminoquinol–HCl was dissolved in 5 ml. of water, the solution was filtered and adjusted to pH 8·0 with 2N-NH_3 soln. to precipitate the free aminoquinol, which was filtered off and redissolved in 2N-HCl. This process was repeated except that the free aminoquinol was finally redissolved in the least quantity of hot 2N-H_2SO_4. On cooling, aminoquinol sulphate separated and this was filtered off and redissolved in 5 ml. of water and acetylated with 1 equiv. of acetic anhydride and excess of sodium acetate. The product was extracted with ether, the ethereal solution was dried over anhydrous Na_2SO_4 and evaporated to dryness to give acetamidoquinol, m.p. and mixed m.p. 100°, which was recrystallized from ether to constant specific activity.

Other possible metabolites

Oxidation products. Aniline is oxidized to nitrosobenzene when it is injected intravenously into dogs (Kiese, 1959*a*, *b*). Phenylhydroxylamine is probably an intermediate in this oxidation and could react with the nitrosobenzene to form azoxy-, azo- and hydrazo-benzene. Further oxidation to nitrobenzene would give rise to *m*- and *p*-nitrophenol (Robinson, Smith & Williams, 1951). The urines were therefore examined for *m*- and *p*-nitrophenol, nitrosobenzene, azoxybenzene, azobenzene and benzidine, since hydrazobenzene forms benzidine on treatment with acids, by the isotope-dilution techniques previously described (Parke, 1956). None of these compounds was found to entrain radioactivity.

Deamination products. The deamination of aromatic amines is unknown, and oxidative deamination of aniline by amine oxidase or by the rat-liver microsomal deaminase (Axelrod, 1955) is most improbable. However, hydrolytic deamination as occurs in the conversion of adenine into hypoxanthine or guanine into xanthine, or reductive deamination similar to that which occurs with mepacrine (Scudi & Jelinek, 1944), might occur with aniline, giving rise to phenol or benzene respectively. The urines were therefore examined for phenol and quinol, the principal metabolites of benzene, by the isotope-dilution procedures previously described (Parke & Williams, 1953). Neither of these compounds was found to entrain radioactivity.

Methylation products. *N*-Methylation is a minor metabolic pathway of the tertiary aromatic bases, pyridine (Tomita, 1921), quinoline and *iso*quinoline (Tamura, 1924). Methylation of a primary aromatic amino group occurs in the metabolism of 4′-fluoro- and 3′-methyl-aminoazobenzene, which, to a small extent, are converted into their *N*-monomethyl derivatives (Miller & Miller, 1952). It was therefore desirable to examine the urines for the *N*-methylation products, methylaniline and dimethylaniline.

Methylaniline (500 mg.) was added to a portion of the urine, which was then refluxed for 3 hr. with 0·5 vol. of conc. HCl; the hydrolysed solution was made alkaline with 5N-NaOH and steam-distilled. The steam-distillate (10 ml.) was acidified with 5 ml. of 2N-HCl and diazotized at 0° by addition of $NaNO_2$ (0·5 g. in 2 ml. of water). The oily nitrosoamine which separated was extracted into ether (three portions of 10 ml.), the extract was washed with water to remove the benzenediazonium sulphate and then dried over anhydrous Na_2SO_4. Evaporation of the ethereal solution gave the *N*-methyl-*N*-nitrosoaniline, which was converted into methylaniline by warming with 5 ml. of a solution of Cu_2Cl_2 in conc. HCl (17·5%, w/v) (Jones & Kenner, 1932). The resulting mixture was diluted with water (10 ml.) and the white precipitate of the copper salt of methylaniline was removed by filtration, washed with a little 2N-HCl, suspended in water and treated with an excess of 2N-NaOH. The methylaniline which was liberated was steam-distilled and the toluene-*p*-sulphonate was prepared, which after recrystallization from ethanol contained no radioactivity (m.p. and mixed m.p. 95°).

Dimethylaniline (500 mg.) was added to a portion of the urine and refluxed for 3 hr. with 0·5 vol. of conc. HCl. The hydrolysed solution was made alkaline with 5N-NaOH and steam-distilled. The dimethylaniline in the steam-distillate was extracted into ether (three portions of 20 ml.), the extract was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was dissolved in 5 ml. of 5N-HCl and treated with a solution of 0·5 g. of $NaNO_2$ in 1 ml. of water. The *p*-nitrosodimethylaniline hydrochloride which separated was filtered off, washed with a little 2N-HCl and suspended in 10 ml. of N-NaOH. The free base which was liberated was collected in ether, the ethereal solution was dried over anhydrous $MgSO_4$ and evaporated to dryness. The residue of *p*-nitrosodimethylaniline had m.p. and mixed m.p. 85° after recrystallization from aq. ethanol and was not radioactive.

Mercapturic acids. Aromatic amines are not known to form mercapturic acids, but the urines were nevertheless examined for the presence of *p*-aminophenylmercapturic acid, *p*-aminothiophenol being used for the isotope-dilution technique, since it was assumed that if aminophenylmercapturic acid was present it would be hydrolysed by alkali to the aminothiophenol. 4:4′-Diaminodiphenyl disulphide (500 mg.) was reduced to *p*-aminothiophenol by refluxing for 15 min. in 10 ml. of ethanol with 2 g. of KOH. A portion of the urine (5–10 ml.) was then added and the mixture was refluxed for a further 1 hr. The solution was then diluted with 10 ml. of water and the ethanol rapidly removed by distillation. After adjusting to pH 6·0 with 2N-H_2SO_4 the solution was steam-distilled and the aqueous distillate of *p*-aminothiophenol was oxidized by the addition of 1 ml. of 20 vol. H_2O_2 and 2 ml. of 2N-NaOH. The product, 4:4′-diaminodiphenyl disulphide, was filtered off and recrystallized from ethanol until finally free from radioactivity (m.p. and mixed m.p. 80°). The rabbit urines were also examined for the presence of mercapturic acids by refluxing for 1 hr. with 0·2 vol. of 10N-NaOH to hydrolyse mercapturic acids to substituted thiophenols. The hydrolysed urines were then adjusted to pH 6·0 with 10N-H_2SO_4 and steam-distilled. The distillates contained no appreciable thiophenol, as determined by iodometric titration, and oxidation with H_2O_2 did not yield any diphenyl disulphide.

Chromatographic detection of metabolites

Aminophenols. The urine from a rabbit dosed with [^{14}C]-aniline (Expt. 3) which remained after the isotope-dilution experiments, was hydrolysed by refluxing with an equal volume of conc. HCl for 3 hr. The hydrolysed solution was cooled, adjusted to pH 8·0 with Na_2CO_3 and continuously extracted with ether for 8 hr. The ethereal extract was dried over anhydrous Na_2SO_4 and chromatographed on paper in wet di*iso*propyl ether (see Table 1). Radioautographs were subsequently developed from the chromatogram before detection of the metabolites by colour tests. The chromato-

Table 2. *R_F values and colour reactions on paper of N-benzoyl derivatives of aminophenols*

R_F values are for descending chromatography on Whatman no. 1 paper. Solvent mixtures: A, $CHCl_3$–formic acid–methanol–water (1000:4:100:96, by vol.) (Reio, 1958) run for 8 hr.; B, benzene–ethyl methyl ketone–formic acid–water (900:100:2:98, by vol.) (Reio, 1958) run for 6 hr. Detecting reagents used were: Gibb's reagent (see Table 1); aq. 0·5% $FeCl_3$ followed by saturated $NaHCO_3$ (pH values are in parentheses); aq. 0·2% soln. of Brentamine Fast Blue 2B salt followed by aq. 2N-NH_3 soln.; aq. 0·2% soln. of Brentamine Fast Garnet GBC salt followed by aq. 2N-NH_3 soln.

	R_F		Colour reactions			
Benzoylated aminophenol	A	B	Gibb's reagent	$FeCl_3$	Brentamine Fast Blue 2B salt	Brentamine Fast Garnet GBC salt
o-Aminophenol	0·93	0·96	Blue	Brown	Yellow	Yellow
m-Aminophenol	0·81	0·89	Violet	None	Orange	Red
p-Aminophenol	0·85	0·98	None	None	Violet	Violet
3-Aminocatechol	0·90	0·96	Blue	Violet (7) Violet (9)	Yellow	Yellow
4-Aminocatechol	0·33	0·46	Blue	Green (7) Violet (9)	Violet	Violet
2-Aminoresorcinol	0·87	0·87	Violet	Pink (7) Buff (9)	Red	Red
4-Aminoresorcinol	0·44	0·80	Violet	Orange (7) Violet (9)	Violet	Red
5-Aminoresorcinol	0·01	0·02	None	None	Violet	Red
Aminoquinol	—	—	None	None	None	None

Table 3. *R_F values and colour reactions on paper of aniline conjugates*

R_F values are for descending chromatography on Whatman no. 1 paper in solvent mixture C, ethanol–butan-1-ol–aq. 3N-NH_3 soln./3N-ammonium carbonate buffer (11:40:19, by vol.) (cf. Fewster & Hall, 1951) run for 16 hr. Detecting reagents were used after hydrolysis by spraying with N-HCl and heating at 100° for 10 min. Aniline was detected with diazo reagent (see Table 1); sulphate was detected with an aq. 0·1% soln. of $BaCl_2$ followed by an aq. 0·1% soln. of rhodizonic acid sodium salt (sulphate gives a white spot on a red background); glucuronic acid was detected by spraying with a 1% soln. of naphtharesorcinol in aq. 20% trichloroacetic acid and heating the paper at 100° for 20–30 min. (glucuronic acid gives a blue spot on a pink background).

		Colour reactions		
Aniline conjugates	R_F	Diazo reagent acid	Naphtha-resorcinol	Rhodizonic acid
Aniline-*N*-glucuronide	0·09	Violet	Blue	None
p-Aminophenyl glucuronide	0·02	Brown	Brown	None
Glucuronic acid	0·02	None	Blue	None
Phenylsulphamic acid	0·26	Violet	None	White
Acetanilide	0·83	Violet	None	None
Aniline*	0·87	Violet	None	None

* Volatile, and therefore only detected when amounts of 200 μg. or more are used.

grams showed six radioactive spots which were identified by R_F values and colour reactions as *p*-aminophenol (R_F 0·39, 50% of dose), *m*-aminophenol (R_F 0·58, 0·1% of dose), *o*-aminophenol (R_F 0·83, 5% of dose), polymeric oxidation products of aminophenols (R_F 0·00, 10% of dose) and unidentified spots at R_F 0·89 (yellow, 0·2% of dose) and R_F 0·95 (pink, 0·1% of dose). Both spots changed to blue–green on spraying with Gibb's reagent. The same spots were also found in chromatograms and radioautographs of ethereal extracts of the hydrolysed urine collected from dogs that had been dosed with [^{14}C]aniline.

Dihydroxyanilines. Urine from a rabbit dosed with [^{14}C]aniline (Expt. 6) which remained from isotope-dilution experiments was hydrolysed with conc. HCl as before and then evaporated to dryness *in vacuo*. The residue was dissolved in 5 ml. of water, treated with 1 equiv. of benzoyl chloride and an excess of sodium acetate and evaporated to dryness *in vacuo* at room temperature. (Free dihydroxyanilines were too susceptible to atmospheric oxidation to permit chromatography.) The residue was extracted with ethanol and the extract chromatographed on paper in solvents A and B (see Table 2). Spots corresponding to *o*- and *p*-aminophenol only were seen, and the aminocatechols and aminoresorcinols were not present in amounts greater than 0·05% of the dose.

Aniline conjugates. The urine from a rabbit that had been dosed with aniline (250 mg./kg.) was collected, for the first day only, in a flask containing 1 ml. of aq. N-NH_3 soln. to

prevent decomposition of aniline conjugates. One-half of the urine (pH 8·0) was treated with an excess of a saturated aq. soln. of basic lead acetate, and the glucuronides were separated as described by Kamil, Smith & Williams (1951). This glucuronide fraction was dissolved in ethanol and chromatographed on paper in solvent C (see Table 3). The other half of the urine was treated with 20 ml. of an aq. 10% (w/v) soln. of $Ba(OH)_2$, the excess of barium was removed with solid CO_2 and the solution was filtered and concentrated to 10 ml. *in vacuo*. The concentrated solution was then treated with 50 mg. of $(NH_4)_2SO_4$, the $BaSO_4$ was filtered off and the filtrate (the phenylsulphamate fraction) was chromatographed on paper in solvent C. Both fractions showed spots corresponding to glucuronic acid (R_F 0·02), aniline-*N*-glucuronide (R_F 0·08, equivalent to 5% of the dose) and phenylsulphamic acid (R_F 0·26, 10% of dose).

RESULTS AND DISCUSSION

The quantitative results for the metabolism of aniline in the rabbit are shown in Table 4. In Expts. 3, 6 and 7 some 80–100% of the dose of radioactivity was accounted for. In 3–8 days after oral dosage with [^{14}C]aniline an average of 70% of the radioactivity is eliminated from the body in the urine: 1% is in the faeces, less than 0·5% is in the expired air and about 5% or more remains in the body, from which it is slowly eliminated via the urine and respiratory carbon dioxide. The urinary excretion of metabolites is fairly rapid; in Expt. 3 over 80% was eliminated in the first 12 hr. after dosing (see Fig. 1); it is usually complete in the first 2 days, but the urines are still slightly radioactive after even 8 days. The radioactivity remaining in the carcass (see Table 5) was evenly distributed throughout the tissues except for a slightly higher concentration in the spleen and fat, and in one case in the gut contents.

Expired air. The radioactivity eliminated in the expired air was very small. Aniline could not be detected within the limits of the method (less than 0·02% of the dose/day) and no other organic metabolites were present. Radioactive carbon dioxide was present in the expired air on the second and subsequent days after dosing, but this amounted to less than 0·4% of the dose in 3 or 4 days (see Fig. 1), although at the termination of the experiments the excretion of $^{14}CO_2$ was still maximal at about 0·2% of the dose/day. Since aniline (b.p. 184°) itself is not excreted in the expired air, it is unlikely to occur in the free state in the blood, for iodobenzene (b.p. 189°) and 1:3:5-trichlorobenzene (b.p. 209°) after oral administration are exhaled in the breath of rabbits to the extent of 3% and 10% of the doses respectively (Azouz, Parke & Williams, 1952; Parke & Williams, 1960).

Metabolites in urine. The metabolites of aniline excreted by the rabbit are shown in Table 4; *p*-

Table 4. *Elimination of metabolites by rabbits receiving* [^{14}C]*aniline*

Doses were given orally. Metabolites were determined in bulked urines collected for 3 days. Tissues were examined after 3, 5 and 8 days in Expts. 3, 6 and 7 respectively. See Table 7 for Expts. 8–10. The S.E.M. is given in parenthesis.

Expt. no.	1	2	3	4	5	6	7	
Dose of aniline (mg./kg.)	160	200	200	250	250	250	500	
Dose of ^{14}C (μc/animal)	9	34	35	41	15	67	109	
Duration of expt. (days)	3	3	3	6	4	5	8	
	Percentage of dose							Average of 10 expts.
Respiratory CO_2	—	—	0·4	—	—	< 0·2	< 0·2	
Aniline (total)	1·2	10·8	19·2*	11·7	6·5	14·5*	4·3*	8·2 (1·8)
Aniline (free)	0·8	2·5	6·4	1·6	—	—	1·0	1·8 (0·7)
Phenylsulphamic acid	—	7·6	8·9	8·2	—	8·0	2·4	5·4 (1·0)
Aniline-*N*-glucuronide	—	4·6	10·8	3·3	—	4·2	1·9	3·5 (1·3)
Acetanilide	0·07	0·2	0·2	—	0·3	0·1	0·3	0·2 (0·03)
o-Aminophenol (total)	5·5	10·1	11·0	9·6	8·4	12·8	9·8	9·1 (0·7)
m-Aminophenol (total)	< 0·1	< 0·1	0·16	—	< 0·1	0·06	0·09	0·1 (0·03)
p-Aminophenol (total)	43	58	66	56	46	47	56	51 (2·6)
p-Aminophenylglucuronide	—	—	9·0	—	—	—	22·2	—
p-Acetamidophenylglucuronide	—	—	17·2	—	—	—	24·6	—
Total metabolites in urine	50	81	97	77	61	72	71	68 (4·7)
Radioactivity in urine	60	80	90	73	69	77	73	70 (4·1)
Radioactivity in faeces	—	—	0·7	—	—	1·1	1·5	—
Radioactivity in tissues	—	—	3·0	—	—	6·3	6·7	—
Total accounted for	—	—	94	—	—	85	81	—

* Less than 0·2% in the expired air.

Table 5. *Occurrence of radioactivity in the tissues of rabbits receiving* [^{14}C]*aniline*

Expt. no.	6			7		
Dose of ^{14}C (μc)	67			109		
Time of death (days after dosing)	5			8		
	Wt. of wet tissue (g.)	Specific activity of tissue (μc/g.)	Percentage of dose present in tissue	Wt. of wet tissue (g.)	Specific activity of tissue (μc/g.)	Percentage of dose present in tissue
Lungs	15	0·001	0·02	14	0·001	0·01
Liver	70	0·001	0·10	53	0·003	0·15
Kidney	11	0·002	0·03	12	0·004	0·05
Heart	7	< 0·001	< 0·01	7	< 0·001	< 0·01
Spleen	2	0·005	0·02	1·5	0·004	0·01
Blood	350	0·002	1·1	350	0·003	1·0
Muscle, voluntary	700	0·001	1·1	750	0·001	0·8
Muscle, involuntary	200	0·001	0·3	200	0·001	0·2
Fat	470	0·004	2·8	550	0·005	2·5
Brain	35	< 0·001	< 0·05	40	0·002	0·8
Ovary	1	0·001	< 0·01	1	< 0·001	< 0·01
Bile	—	—	—	1	< 0·001	< 0·01
Stomach contents	105	0·002	0·3	70	0·005	0·4
Intestinal contents (small intestine)	130	0·002	0·4	210	0·004	0·8
Intestinal contents (caecum)	60	0·002	0·2			
Faeces	75	0·010	1·1	60	0·027	1·5
Total in tissues and faeces	—	—	7·4	—	—	8·2
Total in expired air	—	—	< 0·4	—	—	< 0·4
Total in urine	—	—	77	—	—	73
Total accounted for	—	—	85	—	—	81

Table 6. *Elimination of metabolites in the urines of dogs receiving* [^{14}C]*aniline orally*

Expt. no.	1	2
Dose of aniline (mg./kg.)	175	200
Dose of ^{14}C (μc/animal)	12·5	15
Duration of expt. (hr.)	16*	41*
Aniline (total)	2·8	12·8†
Aniline (free)	0·4	8·9†
Acetanilide	< 0·1	< 0·1
o-Aminophenol	10·7	25·2
m-Aminophenol	< 0·1	< 0·1
p-Aminophenol	7·1	11·3
Sum of metabolites	20·6	49·3
Total radioactivity in urine	18·6	48·5

* Both animals showed symptoms of methaemoglobinaemia and were killed when distressed.

† Some of this was from vomit.

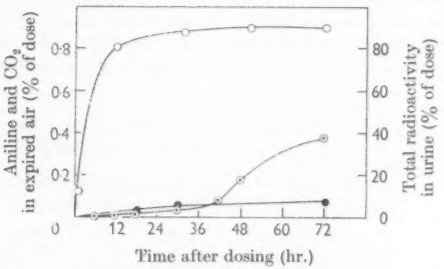

Fig. 1. Excretion of aniline (●) and CO_2 (⊙) in the expired air, and total radioactivity (○) in the urine, of rabbit no. 3 dosed orally with [^{14}C]aniline (200 mg./kg.).

aminophenol accounts for 51 % of the dose. Other metabolites are *o*-aminophenol (9 %), phenylsulphamic acid (5·5 %), aniline-*N*-glucuronide (3·5 %), *m*-aminophenol (0·1 %) and acetanilide (0·2 %). 4-Aminoresorcinol, which was tentatively suggested by Smith & Williams (1949) as a metabolite of aniline, was not found, and none of the other dihydroxyanilines was detected. No anilide other than acetanilide was present and no evidence of methylation or deamination was obtained. There was no evidence of the formation of a mercapturic acid since the isotope-dilution procedure for *p*-aminothiophenol was negative, and steam-distillation of alkali-hydrolysed urines did not yield any thiophenols. No evidence of nitrosobenzene, azoxybenzene, azobenzene or benzidine was found in the urines, but two unidentified metabolites, equivalent to about 0·1 and 0·2 % of the dose, were found by paper chromatography in the urines of both rabbits and dogs. The same two compounds have also been found in chromatograms of the urines of rabbits dosed with nitrosobenzene and with azoxybenzene (D. Robinson, personal communication) and this therefore gives some support to the claim of Kiese (1959*a*, *b*) that aniline is oxidized to nitroso-

benzene. The metabolites of aniline in dog urine have also been estimated (Table 6); 4-aminoresorcinol, phenol, quinol, methylaniline and dimethylaniline were not metabolites.

Aminophenol conjugates. The metabolism of the aminophenols in rabbits was studied by Bray, Clowes & Thorpe (1952*a*), who showed that 4% and 25% of a dose (about 400 mg./kg.), of *o*- and *p*-aminophenol respectively, were excreted as free acetamidophenols. In contrast to this, no free acetamidophenols were found in the urine of rabbits dosed with aniline. Bray *et al.* (1952*a*) also found that 61% of the total *p*-aminophenol was excreted as glucuronides, 45% as *p*-aminophenylglucuronide and 16% as *p*-acetamidophenylglucuronide. In the present experiments the fraction of the total *p*-aminophenol which was excreted as glucuronides varied with the dose. At an oral dose of aniline of 200 mg./kg. rabbits yield 9% of *p*-aminophenylglucuronide and 17% of *p*-acetamidophenylglucuronide, which are equivalent to 14% and 26% of the total *p*-aminophenol. At a dose of 500 mg./kg. the corresponding figures are *p*-aminophenylglucuronide 22% and *p*-acetamidophenylglucuronide 25%, equivalent to 40% and 44% of the total *p*-aminophenol. This is in agreement with Smith & Williams (1949), who, although unable to estimate precisely the total amounts, found by isolation approximately equal amounts of both glucuronides in the urine of rabbits dosed with aniline. This increase of *p*-aminophenylglucuronides with increased dose is in agreement with the increased excretion of glucuronide conjugate at high doses (see Table 7). The principal fact that emerges from this table is that the percentage conjugation with sulphuric acid decreases as the dose increases so that the total amount of aniline metabolites conjugated by this mechanism is approximately constant at 40–70 mg./kg. Glucuronide conjugation on the other hand increases at higher doses. These findings might have been expected from the kinetic studies of Bray, Humphris, Thorpe, White & Wood (1952*b*), who found that the conjugation of phenols with glucuronic acid is a first-order reaction whereas conjugation with sulphuric acid is limited by the availability of sulphate precursors.

Excretion of free glucuronic acid. At all doses the total conjugates are in excess of the total radioactivity in the urine (Table 7), particularly at the toxic dose of 500 mg./kg., when the total conjugates are two to three times as great as the total metabolites. This is probably due to the excretion of free glucuronic acid, which was detected in paper chromatography of the urines, and was in fact isolated by Smith & Williams (1949) from the urines of rabbits dosed with aniline. Free glucuronic acid has been demonstrated in the urines of rabbits dosed with hexachlorobenzene (Parke & Williams, 1960), and in the urines of rats, guinea pigs and dogs dosed with barbital (Burns, Evans & Trousof, 1957), and is probably due to a toxic effect on carbohydrate metabolism. This excretion of free glucuronic acid in aniline urine is responsible for the reducing action of the urines upon Benedict's solu-

Table 7. *Excretion of conjugates and principal metabolites in the urine of rabbits receiving* [^{14}C]*aniline at varying oral doses*

The figures for conjugates are the means for three animals; ranges are in parentheses. The figures for metabolites were obtained by isotope-dilution experiments on one animal only.

Dose of aniline (mg./kg.) ...	100	300	500*
Dose of ^{14}C (μc/animal)	2·9	2·6	2·4
Duration of expt. (days) ...	5	5	3–5
	Percentage of dose		
Glucuronic acid (G)	64 (51–87)	68 (44–86)	122 (98–156)†
Ethereal sulphates‡ (E)	41 (26–50)	17 (10–25)	14 (14–16)
Total conjugates (E + G)	105 (101–113)	85 (69–101)	136 (112–170)
Total radioactivity in urine	80 (77–85)	63 (54–77)	50 (45–60)
Expt. no.	8	9	10
Aniline (total)	4·4	5·0	3·6
Aniline (free)	0·3	1·2	0·5
Aniline-*N*-glucuronide	0·5	1·8	1·1
Phenylsulphamic acid	3·2	2·8	2·2
Acetanilide	0·2	0·2	0·3
o-Aminophenol	8·5	8·5	6·5
p-Aminophenol	52	46	38
Total metabolites in urine	64 (78)§	59 (58)§	48 (45)§

* All animals died from 3 to 5 days after dosing.
† These urines reduced Benedict's solution.
‡ Including phenylsulphamate.
§ Figures in parentheses are for the total radioactivity in the urine.

tion which was observed by Smith & Williams (1949), and was thought to indicate the presence of a labile glucuronide of aniline. In the present experiments, the urines of rabbits dosed with aniline showed a marked reduction of Benedict's solution only at the higher doses (400–500 mg./kg.), i.e. doses at which there is a marked excretion of free glucuronic acid.

Aniline conjugates. The urines of rabbits dosed with aniline contain an average of 8 % of the dose as phenylsulphamic acid (5·5 %) and aniline-*N*-glucuronide (3·5 %). Since one-half of the total aniline of the ammonium salt of aniline-*N*-glucuronide is labile at pH 8·0 (Axelrod, Inscoe & Tomkins, 1958) the estimate of 'free' aniline should be equivalent to half the *N*-glucuronide if this is present in urine as either the ammonium or potassium salt. This is found to be approximately true (see Table 4). The biological syntheses of both aniline conjugates have been effected *in vitro*. Roy (1958) demonstrated the synthesis of phenylsulphamate from aniline by enzyme preparations of guinea-pig and rabbit livers in the presence of adenosine triphosphate and sulphate; and Axelrod *et al.* (1958) demonstrated the transfer of glucuronic acid from uridine diphosphate glucuronic acid to aniline by a microsomal enzyme of guinea-pig liver to yield an *N*-glucosiduronic acid which was identical with the aniline-*N*-glucuronide described in this paper. The acetylation of aniline to acetanilide has been shown to occur in the rabbit, but only a very small amount of acetanilide (0·2 % of dose) was found, which is in keeping with the findings of Smith & Williams (1948) that acetanilide is extensively metabolized to *p*-acetamidophenylglucuronide and *p*-acetamidophenylsulphuric acid.

m-*Aminophenol.* The *m*-aminophenol may arise from direct hydroxylation or from a premercapturic acid, e.g. *N*-acetyl-*S*-(4-amino-1:2-dihydro-2-hydroxy-1-phenyl)cysteine by elimination of *N*-acetylcysteine (see Knight & Young, 1958; Parke & Williams, 1958). The excretion of traces of the *meta* isomer as a metabolite of an aromatic compound with an *ortho/para*-directing substituent is of particular interest since Gessner & Smith (1960) have shown that in addition to *o*- and *p*-chlorophenol, *m*-chlorophenol also occurs in the urine of rabbits dosed with chlorobenzene.

Ratio of p- *to* o-*aminophenol in different species.* The ratio of the total *p*-aminophenol to total *o*-aminophenol was determined in several animal

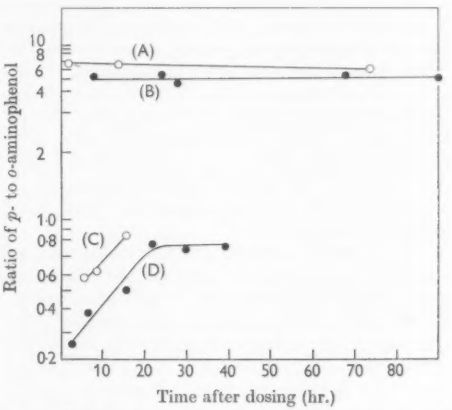

Fig. 2. Ratio of *p*- to *o*-aminophenol excreted in the urines of rabbits and dogs receiving [^{14}C]aniline orally. (A) Rabbit 3 (dose, 200 mg./kg.); (B) rabbit 5 (dose 250 mg./kg.); (C) dog 1 (dose 175 mg./kg.); (D) dog 2 (dose, 200 mg./kg.). The mean *para/ortho* ratios on the bulked urines were: 6·0 for (A), 5·5 for (B), 0·66 for (C) and 0·45 for (D).

Table 8. *Metabolic hydroxylation of aniline by various animal species*

Species	Sex	Oral dose (mg./kg.)	Percentage of dose in 2 days* Total metabolites	Aminophenol *ortho*	Aminophenol *para*	Aniline (total)	Ratio* *para/ortho*	Lethal dose† (g. of aniline/kg. body wt.)
Gerbil	F	250	57	3·2	48	5	15 (12–17)[3]	—
Guinea pig	F	250	55	4·2	46	4·5	11 (9–12)[3]	2·5
Golden hamster	F	250	64	5·5	53	4·5	10 (9–11)[3]	—
Rabbit	F	160–500	71	8·8	50	9	6 (4–9)[10]	1·0
Rat	M	250	52	7·2	42	3	6 (5–6)[3]	—
	F	250	71	19	48	3·5	2·5 (2–3)[3]	—
Chicken	F	50	61	10·5	44	4	4 (4)[2]	—
Mouse‡	M	250	20	4	12	3	3 (2–4)[3]	—
Ferret	F	250	61	26	28	3	1 (1)[2]	—
Dog‡	F	200	33	18	9	8	0·5 (0·4, 0·6)	0·5
Cat‡	F	200	41	32	14	4	0·4 (0·4, 0·5)	0·2

* Figures are mean values; range is given in parenthesis and the number of expts. as indices.
† See Spector (1956).
‡ Animals died or were killed during the first day after dosing.

species with [^{14}C]aniline and was found to vary from 15:1 in the gerbil to 0·4 in the cat (see Table 8). It is significant that in the hydroxylation *in vivo* with liver microsomes of dog, which produces approximately equal amounts of *ortho* and *para* isomers, both isomers were obtained, whereas with liver microsomes from rabbit, which produces an excess of the *para* isomer, only *p*-aminophenol was detected. This increase of *ortho* hydroxylation in the series of animals from gerbil to cat approximately parallels the increase in toxicity of aniline, which is possibly due to *o*-aminophenol being more toxic than the *para* isomer. A similar species difference in hydroxylation has been observed in the metabolism of *N*-2-fluorenylacetamide (Weisberger, Weisberger & Morris, 1957) and β-naphthylamine (Bonser, Clayson & Jull, 1951). Furthermore, the higher production of *ortho*-hydroxylated metabolites in certain species, e.g. dog, has been correlated with a greater incidence of carcinomas, and the carcinogenic activity of aromatic amines may be due to their metabolism to *o*-aminophenols (Clayson, 1953) or to the formation of *iso*phenoxazines by the enzymic oxidation of *o*-aminophenols (Nagasawa & Gutmann, 1959).

One possible explanation for this species difference of the *para*/*ortho* ratio is that the species with a higher ratio, e.g. rabbit, may acetylate the amino group more extensively than species with a lower ratio. The acetanilide so formed would then be hydroxylated preferentially in the *para* position (see Smith & Williams, 1948). A second explanation is that two enzymes are responsible and that these enzymes occur to different extents in different species. From Fig. 2 it can be seen that with the dog the *para*/*ortho* ratio changes markedly during the first day. Since the dog, unlike the rabbit, does not readily acetylate aromatic amino compounds (Marshall, 1954), this change in the *para*/*ortho* ratio could be evidence of the two hydroxylation processes proceeding at different rates.

SUMMARY

1. The fate of [^{14}C]aniline has been studied in the rabbit, dog and other animals.

2. With the rabbit, an average of 70% of the aniline is eliminated in the urine in 3 days as *p*-aminophenol (51%), *o*-aminophenol (9%), *m*-aminophenol (0·1%), phenylsulphamic acid (5·5%), aniline-*N*-glucuronide (3·5%) and acetanilide (0·2%).

3. The isomeric dihydroxyanilines and the free *o*- and *p*-acetamidophenol do not occur in the urine.

4. In 3–4 days the expired air contains no detectable aniline and up to 0·4% of the dose as carbon dioxide. The elimination of carbon dioxide begins on the second day and continues beyond the fourth day.

5. The total excretion of radioactivity in 3 days in the urine, expired air (up to 1%) and faeces (1%) amounts to about 75–90% of the dose. The remainder is present in the tissues and is slowly eliminated as carbon dioxide and as metabolites in the urine. Rabbits killed 5–8 days after dosing contained about 6% of the dose in their tissues. These experiments account for 80–100% of the dose.

6. Free glucuronic acid is excreted in the urine of rabbits after oral administration of aniline, particularly at high doses (500 mg./kg.).

7. With the dog, about 20% of the aniline is eliminated in the urine in 16 hr. and about 50% in 2 days. The principal metabolites are *o*-aminophenol (25%), *p*-aminophenol (11%) and conjugates of aniline (5%).

8. In both rabbit and dog there was no evidence for the *N*-methylation or deamination of aniline, nor for mercapturic acid formation.

9. Nitrosobenzene, azoxybenzene, azobenzene and benzidine, the presence of which would have confirmed the oxidation of aniline to nitrosobenzene, did not occur in the urine. However, two unidentified metabolites, which have also been found in the urines of rabbits dosed with nitrosobenzene or azoxybenzene, were detected in trace amounts by paper chromatography.

10. The ratio of *p*- to *o*-aminophenol excreted in the urines of various animal species dosed with aniline was: gerbil, 15; guinea pig, 11; golden hamster, 10; rabbit, 6; rat, 6 (male) and 2·5 (female); chicken, 4; mouse, 3; ferret, 1; dog, 0·5; cat, 0·4.

11. With the dog, the *para*/*ortho* ratio changes with time and it is suggested that at least two different enzyme systems are involved in the hydroxylation of aniline.

I am indebted to Professor R. T. Williams for his constant interest in this work.

REFERENCES

Axelrod, J. (1955). *J. biol. Chem.* **214**, 753.
Axelrod, J., Inscoe, J. K. & Tomkins, G. M. (1958). *J. biol. Chem.* **232**, 835.
Axelrod, J., Udenfriend, S. & Brodie, B. B. (1954). *J. Pharmacol.* **111**, 176.
Azouz, W. M., Parke, D. V. & Williams, R. T. (1952). *Biochem. J.* **50**, 702.
Baldwin, B. C., Robinson, D. & Williams, R. T. (1960). *Biochem. J.* **76**, 600.
Bergmann, M. & Wolff, W. W. (1923). *Ber. dtsch. chem. Ges.* **56**, 1060.
Bonser, G. M., Clayson, D. B. & Jull, J. W. (1951). *Lancet*, ii, 286.
Booth, J. & Boyland, E. (1957). *Biochem. J.* **66**, 73.
Boyland, E., Manson, D. & Orr, S. F. D. (1957). *Biochem. J.* **65**, 417.

Braude, E. A., Linstead, R. P. & Wooldridge, K. R. H. (1954). *J. chem. Soc.* p. 3586.
Bray, H. G., Clowes, R. C. & Thorpe, W. V. (1952*a*). *Biochem. J.* **51**, 70.
Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1952*b*). *Biochem. J.* **52**, 416.
Brodie, B. B., Axelrod, J., Shore, P. A. & Udenfriend, S. (1954). *J. biol. Chem.* **208**, 741.
Burns, J. J., Evans, C. & Trousof, N. (1957). *J. biol. Chem.* **227**, 785.
Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. H. & Yankwich, P. F. (1949). *Isotopic Carbon*. New York: John Wiley and Sons Inc.
Clayson, D. B. (1953). *Brit. J. Cancer.* **7**, 460.
Fewster, M. E. & Hall, D. A. (1951). *Nature, Lond.*, **168**, 78.
Gessner, T. & Smith, J. N. (1960). *Biochem. J.* **75**, 172.
Henrich, F. (1921). *Ber. dtsch. chem. Ges.* **54**, 2507.
Henrich, F. & Wagner, B. (1902). *Ber. dtsch. chem. Ges.* **35**, 4200.
Heyns, K. & Baltes, W. (1958). *Ber. dtsch. chem. Ges.* **91**, 622.
Hinsberg, O. (1906). *Ber. dtsch. chem. Ges.* **39**, 2427.
Hodgson, H. H. & Dix, A. G. (1914). *J. chem. Soc.* **105**, 954.
Huennekens, F. M., Osborn, M. J. & Whiteley, H. R. (1958). *Science*, **128**, 120.
Ishidate, M., Hogiya, S., Hashimoto, Y. & Takitani, S. (1958). *Pharm. Weekbl.* **93**, 216.
Jacobs, W. A., Heidelberger, M. & Rolf, I. P. (1919). *J. Amer. chem. Soc.* **41**, 467.
Jones, E. C. S. & Kenner, J. (1932). *J. chem. Soc.* p. 711.
Kamil, I. A., Smith, J. N. & Williams, R. T. (1951). *Biochem. J.* **50**, 235.
Kauffmann, H. & dePay, E. (1906). *Ber. dtsch. chem. Ges.* **39**, 323.
Kehrmann, F. & Bahatrian, G. (1898). *Ber. dtsch. chem. Ges.* **31**, 2400.
Kehrmann, F. & Hoehn, E. (1925). *Helv. chim. acta*, **8**, 222.
Kiese, M. (1959*a*). *Arch. exp. Path. Pharmak.* **235**, 354, 360.
Kiese, M. (1959*b*). *Arch. exp. Path. Pharmak.* **236**, 19.
Knight, R. H. & Young, L. (1958). *Biochem. J.* **70**, 111.
Marshall, E. K., jun. (1954). *J. biol. Chem.* **211**, 499.
Mead, J. A. R., Smith, J. N. & Williams, R. T. (1958). *Biochem. J.* **68**, 61.
Miller, J. A. & Miller, E. C. (1952). *Cancer Res.* **12**, 283.
Mitoma, C., Posner, H. S., Reitz, H. C. & Udenfriend, S. (1956). *Arch. Biochem. Biophys.* **61**, 431.
Nagasawa, H. T. & Gutmann, H. R. (1959). *J. biol. Chem.* **234**, 1593, 1600.
Parke, D. V. (1956). *Biochem. J.* **62**, 339.
Parke, D. V. & Williams, R. T. (1953). *Biochem. J.* **54**, 231.
Parke, D. V. & Williams, R. T. (1958). *Rep. Progr. Chem.* **55**, 386.
Parke, D. V. & Williams, R. T. (1960). *Biochem. J.* **74**, 5.
Pollack, J. (1893). *Mh. Chem.* **14**, 419.
Reio, L. (1958). *J. Chromat.* **1**, 338.
Robinson, D., Smith, J. N. & Williams, R. T. (1951). *Biochem. J.* **50**, 228.
Roy, A. B. (1958). *Biochim. biophys. Acta*, **30**, 193.
Sato, T., Suzuki, T., Fukuyama, T. & Yoshikawa, H. (1955). *Seitai no Kagaku*, **6**, 225. (*Chem. Abstr.* **52**, 20534).
Scudi, J. V. & Jelinek, V. C. (1944). *J. biol. Chem.* **152**, 27.
Smith, J. N. & Williams, R. T. (1948). *Biochem. J.* **42**, 541.
Smith, J. N. & Williams, R. T. (1949). *Biochem. J.* **44**, 242.
Spector, W. S. (1956). *Handbook of Toxicology*, vol. 1, p. 26. London: W. B. Saunders Co. Ltd.
Spicer, S. S. (1950). *J. Pharmacol.* **99**, 185.
Tamura, S. (1924). *Acta Sch. med. Univ. Kioto*, **6**, 449, 459.
Thierfelder, H. (1889). *Hoppe-Seyl. Z.* **13**, 277.
Tomita, M. (1921). *Biochem. Z.* **116**, 55.
Traube, W. (1890). *Ber. dtsch. chem. Ges.* **23**, 1654.
Volkova, S. A. (1958). *J. Hyg. Epidem. Microbiol. Immunol., Prague*, **2**, 375.
Weisberger, J. H., Weisberger, E. K. & Morris, H. P. (1957). *Science*, **125**, 503.
Williams, R. T. (1943). *Biochem. J.* **37**, 329.
Williams, R. T. (1959). *Detoxication Mechanisms*, 2nd ed. p. 13. London: Chapman and Hall Ltd.

Biochem. J. (1960) **77**, 503

The Chemical Composition and Structure of the Cell Wall of *Hydrodictyon africanum* Yaman

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The coenobium of the green alga *Hydrodictyon africanum* consists in a single net of about 500 cells or coenocytes of similar size and stage of development. The net-like colonies can easily be grown in culture, the individual cells reaching a size of 2 mm. or more in diameter. The organism thus gives material which is very well suited to a study of plant cell growth and in particular to the development of the cell wall. The walls are large and can be obtained free from the cell contents by a variety of techniques including microdissection, which is both mechanically and chemically very gentle. The work reported here is a microscopical and chemical study of the cell walls prepared from fully developed cells.

The species *H. africanum* has been described by Yamanouchi (1913), Wigglesworth (1928) and Pocock (1937). *Hydrodictyon reticulatum* has been grown under laboratory conditions and used in photosynthesis and other studies (Neeb, 1952; Schön, 1955; Pirson & Schön, 1957; Pirson, 1957).

EXPERIMENTAL

Material used and general analytical methods

Organism. Hydrodictyon africanum Yaman. (Culture no. 236/2) was originally obtained from the culture collection of algae and protozoa in the Botany School, Cambridge, and was kindly given by Mr E. A. George.

The nets were grown in a sterile soil extract prepared in 1 lb. Kilner jars (500 ml.) without the metal cap or rubber ring. The soil (approx. 50 g.), which was collected from the gardens near the laboratory, was mixed with $CaCO_3$ (approx. 0·4 g.) and covered with 420 ml. of tap water and steamed for 1 hr. It was allowed to cool to room temperature and steamed again for a further hour. The medium was used in the jars, as prepared, without removal of the soil which settled after about a week. The jars were stored for about a month before use when the liquid above the soil was a clear light amber colour and contained 0·13 g. of solid matter/l. For the germination of zygospores a similar medium was prepared but the soil used was Kettering loam.

The zygospores were kept in media in which the nets had developed and had produced gametes. After the collapse of the net the zygospores could be seen as an orange layer on top of the soil: in this form they could be stored for at least 3 years. The spores were germinated by transferring them to a fresh medium kept at 18–20° in a light intensity of 600 lux for periods of 16 hr. followed by 8 hr. of darkness. The light was supplied by two 40 w, 2 ft. natural fluorescent tubes and two 40 w tungsten filament lamps. After 3–4 weeks small green nets approx. 1–2 mm. in diameter could be seen and when these were transferred, one net to a jar, and grown under the same conditions as that used for germination, they developed into large nets 5–10 cm. across in which the individual cells had a diameter of 1–3 mm. Growth to this size usually took about 4 weeks. Each net normally consisted of about 250 or 500 cells (approx. 2^8 or 2^9) and some colonies with up to 1000 cells were obtained.

Isolation of the cell-wall material by mechanical breakage of the cells. The nets were removed from the medium and washed in distilled water and the individual cells separated by gently pushing the cells apart with a small spatula. Generally any one cell was round and taut and joined to four other cells, two at one pole and two at the other (Pl. 1, fig. 2 inset). The cell was broken with a hypodermic needle and the contents were washed out by injecting distilled water. The process was watched under a microscope (× 12·5) and was continued until no cell contents could be seen adhering to the wall. The wall appeared as a transparent colourless membrane which was transferred with fine forceps to a beaker of water and washed by gentle agitation. The damp walls were freeze-dried.

All the analyses were carried out on material dried at 0·01 mm. Hg over P_2O_5 at room temperature. Total N was determined by micro-Kjedahl digestion (Chibnall, Rees & Williams, 1943) followed by distillation and titration. Total P was determined according to Fiske & Subbarow (1925).

Chromatography and electrophoresis of sugars. Descending chromatograms were run on Whatman no. 1 papers with pyridine–ethyl acetate–water (1:2:2, by vol.) for 14 hr. and ethyl acetate–acetic acid–water (3:1:3, by vol.) for 24 hr. (Jermyn & Isherwood, 1949). Electrophoreses were run at 210 v in an apparatus similar to that described by Consden & Stanier (1952) with borate buffer, pH 9·2. The sugar spots were coloured in all procedures with aniline hydrogen phthalate (Partridge, 1949).

Chromatography of amino acids. Two-dimensional chromatograms were run on Whatman no. 52 paper with butanol–acetic acid–water (4:1:5, by vol.) and phenol–*m*-cresol (1:1, v/v) buffered at pH 9·3 with borate according to Levy & Chung (1953). The spots were coloured with a 0·3% soln. of ninhydrin in butanol.

Chemical investigation of the cell wall

The average dry weight of one cell was 26 μg. (833 cells, wt. 21·9 mg.) and the cell wall was 10·2 μg. (616 walls, wt. 6·3 mg.), 39·2%. The cytoplasm of the cell could be seen as a thin layer of granular material closely applied to the cell wall [Pl. 1, figs. 2 (inset) and 5] and inside this, occupying the bulk of the cell volume, was the large vacuole.

Elementary analysis and mineral content. The total N was 0·65% and the total P 0·168% of the dry weight. When maintained at red heat (approx. 800°) in a platinum boat in a stream of clean dry air for 1 hr., 17·23 mg. of walls yielded a white ash; 0·05 mg. (0·3%).

Lipid analysis. The lipid was determined by boiling the cell walls (57·3 mg.) with aq. 80% (v/v) methanol (20 ml.) for 2·5 hr. and centrifuging and siphoning off the alcoholic supernatant from the walls, which were redried at 0·01 mm. Hg over P_2O_5. The material was re-extracted with boiling dry ether (20 ml.) for 2·5 hr. Yield of extracted cell walls was 53·2 mg. and the wt. loss of the walls was 4·1 mg. (7·2%). The methanol and ether extracts were combined and evaporated under reduced pressure to a thick yellow oil (3·8 mg.).

Acid hydrolysis. (i) *For monosaccharide constituents.* The whole cell walls (5 mg.) were suspended in 0·1 ml. of 72% (w/v) H_2SO_4 and left at room temperature for 24 hr. when most of the material dissolved. The solution was diluted with water (0·7 ml.) and heated at 95° for 6 hr. in a sealed tube, then neutralized with $BaCO_3$, filtered, and the filtrate was evaporated to dryness at 0°. Galacturonic acid, glucose, mannose, xylose and a very slight trace of arabinose were found in the hydrolysate by chromatographic and electrophoretic investigations.

(ii) *For amino acids.* Whole cell walls (7 mg.) were hydrolysed by 6N-HCl (3 ml.) at 105° for 24 hr. in a sealed tube. The hydrolysate was evaporated at 25° under vacuum, dissolved in water and re-evaporated

EXPLANATION OF PLATES 1–3

In all Figs.: *o*, outer edge of wall; *i*, inner edge of wall; *l*, laminations; *p*, pores; *pm*, pore membrane; *m*, microfibrils.

PLATE 1

Figs. 2, 3. Whole cell wall stained with light green. The circular pads at the poles of the cell can be seen.

Fig. 2 (inset). Portion of a whole colony showing the arrangement of the cells.

Fig. 4. Portion of cell wall treated with 0·5% NaOH at 4° for 24 hr. Shadowed with Pd/Au at an angle of 60°. The microfibrillar organization can be seen.

Fig. 5. Transverse section of a whole small cell (1 mm. in diameter) fixed in osmic acid. The three layers of the wall are visible as a thin outer and inner membrane of dark staining material and a thick middle layer hardly stained.

PLATE 2. All the sections are cut transverse, see Fig. 1 (p. 506).

Fig. 6. Wall treated with 3% NaOH for 24 hr. and stained with osmic and phosphotungstic acids. A pore can be seen passing obliquely across the wall. The dark thin outer layer shows very clearly and in the middle layer there are indications of the thin laminations running parallel to the cell surface.

Fig. 7. Untreated wall fixed with osmic acid. The membrane of the pore passing straight across the section appears to be continuous with the inner and outer layers of the wall.

Fig. 8. Wall treated with 3% NaOH for 24 hr. and fixed in osmic acid. A detailed view of a pore at the inner surface of the wall is shown.

Fig. 9. Untreated wall fixed with osmic acid. A branched pore is visible.

PLATE 3

Fig. 10. Section of untreated wall fixed in osmic acid and cut tangentially (see Fig. 1). Fine microfibrils (30–40 Å in diameter) can be seen as closely packed structures running parallel to the edge of the section. A lamination runs across the section at right angles to the microfibrils. The pores can be seen as holes bounded by thin membranes.

Fig. 10 (inset). Microfibrillar structure at higher magnification.

Fig. 11. Untreated wall fixed in potassium permanganate. The fine laminations of the middle layer can be clearly seen. The outer edge of the wall is formed by the darkly stained material and the inner layer appears granulated.

(*Facing p.* 504)

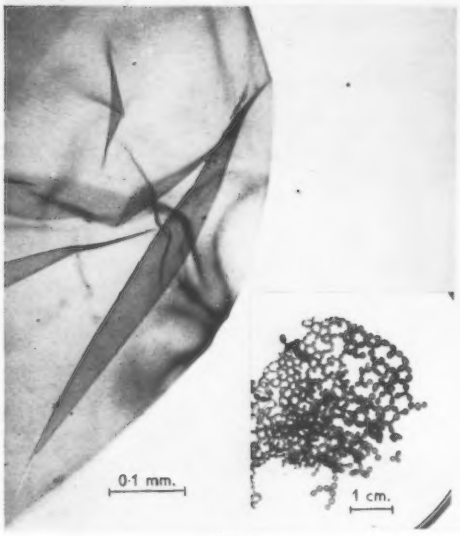

Fig. 2

Fig. 3

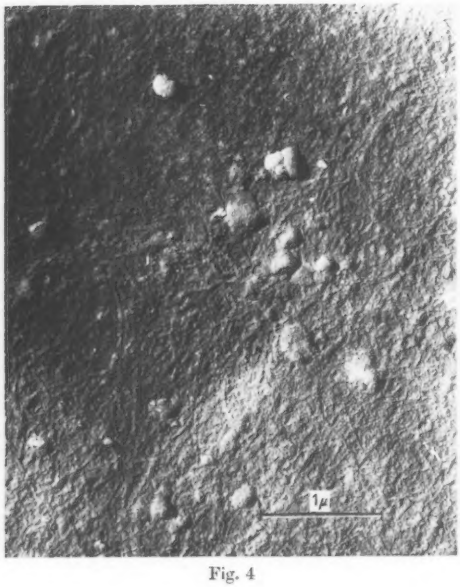

Fig. 4

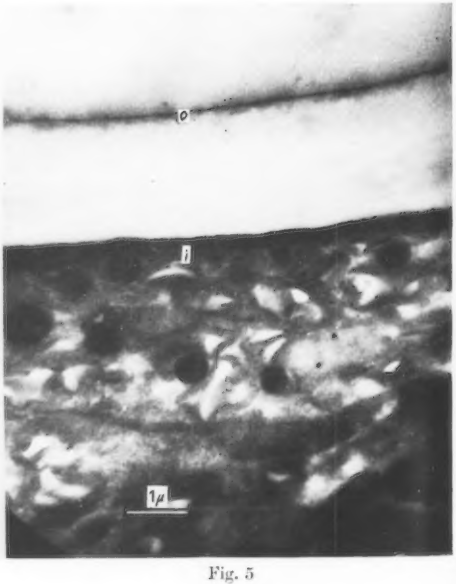

Fig. 5

Fig. 6

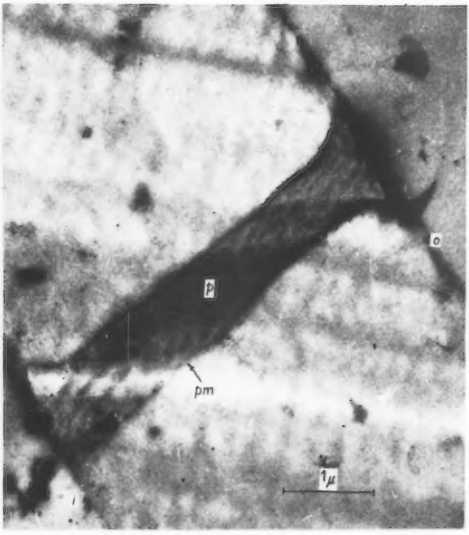

Fig. 7

Fig. 8

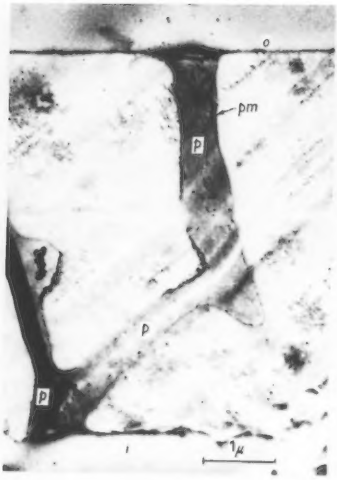

Fig. 9

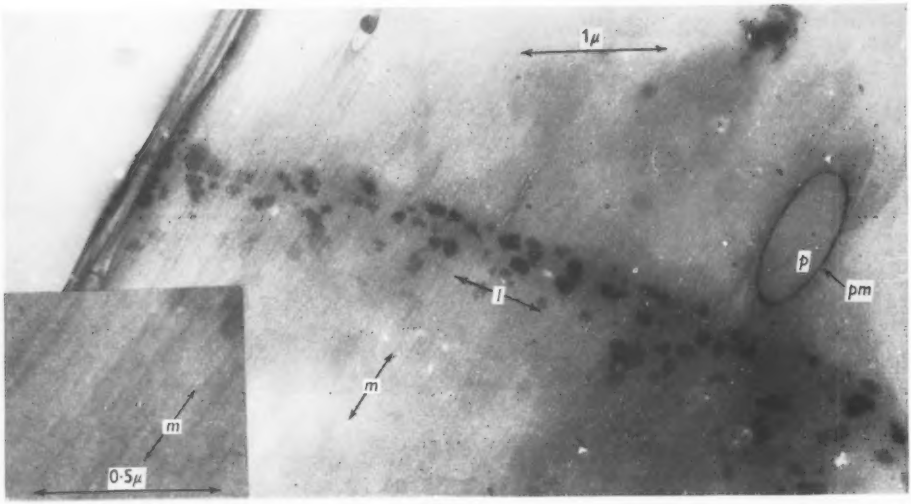

Fig. 10

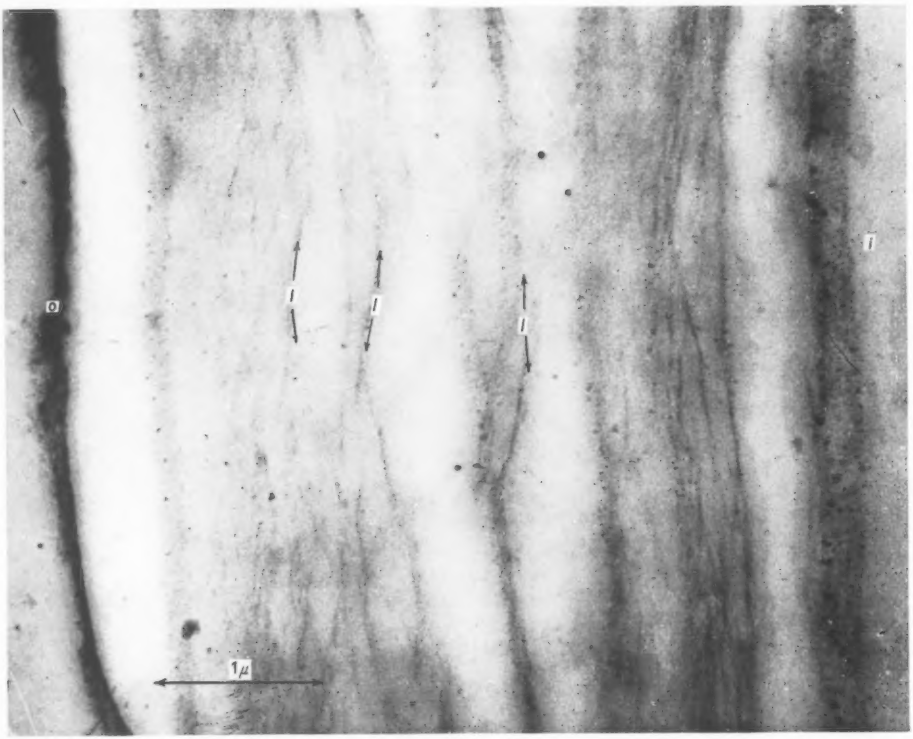

Fig. 11

several times. Aspartic acid, glutamic acid, serine, glycine, alanine, tyrosine, valine, leucine/isoleucine, lysine and smaller amounts of threonine, phenylalanine, proline, hydroxyproline, arginine and cysteine were identified in the hydrolysate.

Polysaccharides of the cell wall. Walls (53·2 mg.) from which lipid had been extracted were treated with 24 % (w/v) KOH (10 ml.) at room temperature under N_2 for 4 hr. The residue from the extraction was well washed with water by suspension and centrifuging. Six washings were required to give a supernatant with the same pH value as the water used for washing. The washings and alkaline extract were combined and the residual α-cellulose fraction was dried over P_2O_5 at 0·01 mm. Hg. Yield of white translucent material was 39·4 mg. (68·7 %). It still retained the shape of the original cell wall.

The alkaline solution of the hemicellulose fraction was neutralized at 4° with cooled acetic acid and evaporated under reduced pressure to half its volume. It was poured into a volume of ethanol so that the final concentration of alcohol was 85 %. A precipitate did not appear immediately but after 2 hr. a discrete gelatinous precipitate formed and settled. It was left for 24 hr. when it was centrifuged, washed with ethanol and dried. Yield of slightly brown coloured material was 9·0 mg. (15·7 %).

The α-cellulose fraction (6 mg.) was hydrolysed with 72 % (w/v) H_2SO_4 according to the method of Saeman, Moore, Mitchell & Millett (1954), and a chromatogram of the neutralized hydrolysate showed glucose and mannose only. These sugars were estimated in the mixture by the method of Wilson (1959) and the molar ratio was found to be 1:1. Even preparing an α-cellulose fraction from the walls with 24 % (w/v) KOH + 4 % (w/v) boric acid (Jones & Painter, 1957), or by boiling with 10 % (w/v) NaOH in the absence of air for 4 hr., did not appreciably alter the ratio of glucose to mannose in the preparation.

The hemicellulose fraction when hydrolysed with 2N-H_2SO_4 at 100° for 6 hr. and neutralized with an ion-exchange resin (Amberlite IR-4B, CO_3^{2-} form) contained glucose, mannose with trace amounts of xylose and very slight traces of arabinose. The glucose and mannose were the main sugars in the hydrolysate and these were present in the molar ratio of 2:3 respectively. Galacturonic acid could be detected in the material when the hydrolysis was carried out with 2N-H_2SO_4 or 3 % HNO_3 and the solution neutralized with $BaCO_3$. The hemicellulose solution did not colour with I_2 solution and contained no starch.

This hemicellulose fraction was investigated by zone electrophoresis on glass paper with borate buffer, pH 9·2 (Fuller & Northcote, 1956). One spot was observed at a distance of 3·5 cm. from the origin towards the cathode, 2:3:6-tri-*O*-methyl-D-glucose moved 13 cm. and starch used as a marker gave a spot at 6·4 cm.

Microscopical examination of the cell wall

The cell walls were examined by the optical and electron microscopes. The cell walls were seen as colourless transparent bags which were easily stained by methylene blue, zinc iodide chloride and light green (Pl. 1, figs. 2, 3). After the cell had been emptied of its contents four circular thin pads could be seen situated in pairs at opposite poles on the outside of the walls and corresponding to the positions where the cell had been in contact with its neighbours in the intact colony (Pl. 1, figs. 2, 3). These pads were about 110 μ in diameter for a cell approx. 2 mm. in diameter (six cells measured, size range 110–111 μ) and in optical section seemed to be slightly raised at the edges (Pl. 1, fig. 2).

A comprehensive examination of the cell wall was made under the electron microscope with a Siemens Elmiskop operating at a high-tension voltage of 80 kv and also with a Phillips E.M. 100 operating at a high-tension voltage of 60 kv. The preparations were supported on nitrocellulose and 'Formvar' (polyvinyl formal) films stabilized with evaporated carbon, which were mounted on copper grids (New 200, diam. 2·3 and 3 mm.; V. A. Howe and Co. Ltd., London, W. 11).

The whole wall was too large to be directly observed with the electron microscope and was broken into small pieces by suspension in water in a small blender (3 ml.) driven at 14 000 rev./min. by a top drive (M.S.E. Ltd., London, S.W. 1) for 10 min. The pieces of wall were centrifuged, resuspended in water and allowed to dry at room temperature in air on to the specimen grids. These were shadowed with Fd/Au, by using standard shadow-casting techniques. The shadowing angle was 60°.

Sectioned material was prepared by fixing the wall in buffered (pH 7·3) osmium tetroxide solution (1 %, w/v) (Palade, 1952) or $KMnO_4$ (0·6 %, w/v) (Luft, 1956). In some instances the preparations stained with osmium tetroxide were further stained by leaving them for 24 hr. in a solution of phosphotungstic acid (1 %, w/v) in 70 % ethanol during the dehydration procedure (Huxley, 1957). The specimens after washing and dehydration were embedded in a mixture of 85 % (v/v) of butyl methacrylate and 15 % (v/v) methyl methacrylate which was polymerized with 1·5 % (w/v) of benzoyl peroxide at 50°. The sections were cut with glass knives in a microtome described by Porter & Blum (1953).

Unsectioned material. Microfibrils could not be seen in untreated preparations of the cell wall but were apparent when the wall fragments were treated with 0·5 % (w/v) NaOH at 4° for 24 hr. (Pl. 1, fig. 4). The microfibrils were closely packed and in

any fragment examined the majority ran in one direction but there were always some which appeared to be oriented across this preferred direction. They could not be obtained free from other wall material.

Sectioned material. Both untreated cell walls and those which had been soaked for 24 hr. with 3% NaOH at room temperature were used. There were no obvious differences in the two preparations (Pls. 1 and 2, figs. 5–9). The sections of the whole cell and the cell wall (Pls. 1 and 2, figs. 5–9) stained with osmium tetroxide showed that the cell wall consisted of three main layers, a thin outer dark staining area approx. 250 Å thick, a middle section 5 μ across which hardly stained, and an inner layer of darker staining material which varied in thickness between approx. 200 and 1000 Å (Pl. 2, figs. 6–9). In the sections stained with osmium tetroxide there were some indications of laminations (approx. 250 Å thick) parallel to the outer and inner surfaces of the wall in the thick middle section (Pl. 2, fig. 6) and these became very apparent when the material was stained with permanganate (Pl. 3, fig. 11). Occasionally sections were seen in which the laminations ran across the section at right angles to those more commonly observed (Pl. 3, fig. 10).

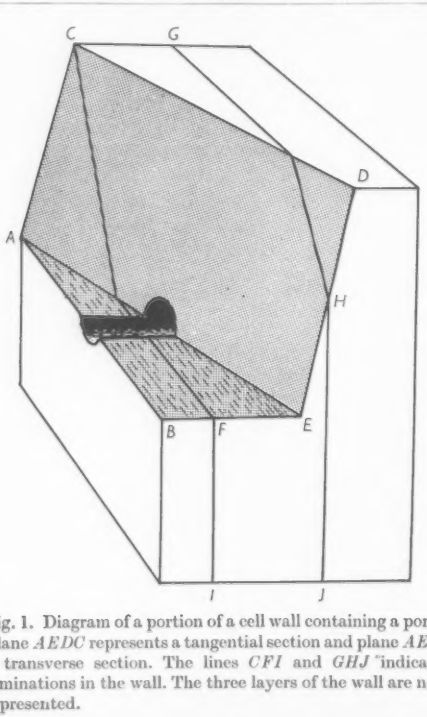

Fig. 1. Diagram of a portion of a cell wall containing a pore. Plane *AEDC* represents a tangential section and plane *AEB* a transverse section. The lines *CFI* and *GHJ* indicate laminations in the wall. The three layers of the wall are not represented.

From other evidence, which is discussed below and which relates to pores seen in the wall, there is reason to believe that these latter sections were tangential. In Fig. 1 plane *ABE* represents a transverse section where the laminations run parallel to the inside and outside of the wall, and plane *AEDC* represents a tangential section in which the laminations (lines *CFI* and *GHJ*) appear across the section more or less at right angles to those in plane *ABE*. The sections cut in plane *AEDC* showed very detailed microfibrillar structures closely packed and all running in one direction around the wall. The diameter of the microfibrils in these stained preparations was 30–40 Å (Pl. 3, fig. 10).

In most sections of the wall dark-staining strips of tissue (diameter 0·7 μ) bounded by a thin membrane 200 Å thick, could be seen passing across the wall from the inside to the outside of the cell (Pl. 2, figs. 7 and 9). Sometimes they appeared to run very obliquely (Pl. 2, fig. 6) and some were branched (Pl. 2, fig. 9), but more usually they were unbranched and passed fairly straight across the wall. The membrane bounding the strip could be seen in some cases to be continuous with the layers at the inside and outside of the wall (Pl. 2, figs. 7–9) and this suggested that the structures were pores containing material which had a strong affinity for osmium tetroxide or that their dark appearance was due to the obvious membrane structure around them which took up the osmium very strongly. The idea that these structures were pores was further strengthened by the appearance of holes in the sections cut in the plane *AEDC* (Fig. 1). These can be seen in Pl. 3, fig. 10, to be oval (diameters 1 $\mu \times 0{\cdot}4\ \mu$) and bounded by a thin strongly staining membrane. The microfibrillar structure can be seen to bend around these pores like the graining of wood around a knot hole.

DISCUSSION

The cell wall of this alga is a large structure representing over 39% (w/w) of the dry weight of the cell and measuring approximately 5·5 μ thick. It is composed largely of α-cellulose-like material (69%) which retains the shape and structure of the original wall and is organized as a microfibrillar phase. The microfibrils are very fine (about 30–40 Å in diameter) and are closely packed and run in a definite direction around the wall. However the α-cellulose-like material occurs as layers which lie between thin laminations running parallel with the cell surface, and the direction of the microfibrils, although seemingly constant in any one layer, may be different in the different layers. The size of the microfibrils in this green alga is similar to that of *Chlorella pyrenoidosa* (Northcote, Goulding & Horne, 1958). Generally in higher plants the

microfibrils are larger, ranging from about 50 to 250 Å in diameter (Northcote, 1958).

The lipid (7 %) which has a greater affinity for osmium than the rest of the material appears to be situated as thin layers both at the inside and outside of the wall and also as membranes for pores which traverse it.

The hemicellulose material (16 %) and protein (4 %, calculated from the nitrogen content) can be removed from the wall without noticeably affecting its structure, and they therefore probably constitute the matrix material around the microfibrils and might also be concentrated to form the thin laminations which divide the α-cellulose-like material. On hydrolysis the hemicellulose material gives glucose and mannose with small amounts of xylose and arabinose which indicate that the principal polysaccharide present is a glucomannan. The α-cellulose fraction also contains mannose in an amount equal to that of the glucose present. In the chemistry of wood the occurrence of these non-glucose sugars in α-cellulose preparations is well known (Bertrand, 1899; Norman, 1937; Northcote, 1958; Polglase, 1955; Wise, 1958). Recently, however, the mannose polymers have been progressively removed from the preparations by means of various alkaline solutions (Hamilton & Quimby, 1957) and mixtures of alkali and boric acid (Jones & Painter, 1957); and Rapson & Morbey (1959) claim to have obtained a highly pure cellulose from wood, that is an α-cellulose preparation comparatively undegraded but almost free of non-glucose sugars. On the other hand, Cronshaw, Myers & Preston (1958) have reported that an α-cellulose preparation obtained in small yield from the whole cells of a marine alga, *Porphyra*, contained mannose only. It is, however, very difficult to detect small amounts of one sugar in the presence of large amounts of others. In addition the presence of non-glucose sugars in α-cellulose material can be explained in two ways: (*a*) as contaminants from the hemicellulose of the continuous matrix surrounding and adhering to fibrils, or (*b*) as part of the polymer(s) forming the microfibrillar structure (Northcote, 1958). The very close packing of the microfibrils in *Hydrodictyon* and the difficulty of obtaining a microfibrillar preparation, which when examined by the electron microscope appeared free from matrix material, makes an explanation for the occurrence of mannose in this α-cellulose impossible at present.

Galacturonic acid is present in the hydrolysates of the whole wall, and this is probably derived from pectic substances. In the higher plant these materials are found in the middle lamella between adjacent cells, and in *Hydrodictyon* it might well be present in the thin circular pads which occur between neighbouring cells in the colony and which can be clearly seen when the walls have been freed from cell contents.

The pores bounded by a membrane are the most obvious feature in the sections, and since the wall is a very large structure in this plant their function is most probably that of transport. However they cannot be open holes from the outside to the vacuole since the osmotic pressure of the cell is such that when pricked it usually bursts. For a similar reason there can be no direct open connexion between the cells of a colony, for they can easily be separated without any obvious change.

SUMMARY

1. Cell-wall preparations of the coenocytes of *Hydrodictyon africanum* have been obtained. The walls are approximately 5·5 μ thick and represent 39·2 % of the dry weight of the whole cell.

2. An analysis of the wall has been made with respect to the following fractions: α-cellulose fraction (69 %), hemicellulose fraction (16 %), protein (4 %), lipid (7 %) and ash (0·3 %), which accounts for over 96 % of the material.

3. The cell wall appears as a transparent colourless membrane and two pairs of thin circular pads, approximately 110 μ in diameter, can be seen at opposite poles corresponding to the position of neighbouring cells in the intact colony. The walls stain with the normal histochemical tests for cellulose.

4. The wall is made up of three layers, thin inner and outer layers and a thick middle layer. The middle layer is a two-phase system in which microfibrils approximately 30–40 Å in diameter are closely packed and enclosed in a continuous matrix. This layer is further divided by very thin lamellae which run parallel to the cell surface, and the microfibrils in any one section of it lie mainly in one direction around the wall.

5. The hemicellulose fraction contains glucose, mannose and traces of xylose and arabinose. It is probably present as a glucomannan. The α-cellulose fraction is composed of polymers containing glucose and mannose. The occurrence of the mannose in the α-cellulose fraction with respect to the composition of the microfibril is discussed.

6. Pores seen as oval in shape (1 μ × 0·4 μ in diameter) which are bounded by a dark-staining membrane traverse the wall.

REFERENCES

Bertrand, G. (1899). *C.R. Acad. Sci., Paris*, **129**, 1025.

Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.

Consden, R. & Stanier, W. M. (1952). *Nature, Lond.*, **170**, 1069.

Cronshaw, J., Myers, A. & Preston, R. D. (1958). *Biochim. biophys. Acta*, **27**, 89.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Fuller, K. W. & Northcote, D. H. (1956). *Biochem. J.* **64**, 657.
Hamilton, J. K. & Quimby, G. R. (1957). *T.A.P.P.I.* **40**, 781.
Huxley, H. E. (1957). *J. biophys. biochem. Cytol.* **3**, 631.
Jermyn, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.
Jones, J. K. N. & Painter, T. J. (1957). *J. chem. Soc.* p. 669.
Levy, A. L. & Chung, D. (1953). *Analyt. Chem.* **25**, 396.
Luft, J. H. (1956). *J. biophys. biochem. Cytol.* **2**, 799.
Neeb, O. (1952). *Flora, Jena*, **139**, 39.
Norman, A. G. (1937). *The Biochemistry of Cellulose, the Polyuronides, Lignin, etc.* Oxford University Press.
Northcote, D. H. (1958). *Biol. Rev.* **33**, 53.
Northcote, D. H., Goulding, J. K. & Horne, R. W. (1958). *Biochem. J.* **70**, 391.
Palade, G. E. (1952). *J. exp. Med.* **95**, 285.
Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
Pirson, A. (1957). *Research in Photosynthesis*, p. 490. New York: Interscience Publishers Inc.
Pirson, A. & Schön, W. J. (1957). *Flora, Jena*, **144**, 447.
Pocock, M. A. (1937). *Trans. Roy. Soc. S. Afr.* **24**, 263.
Polglase, W. J. (1955). *Advanc. Carbohyd. Chem.* **10**, 283.
Porter, K. R. & Blum, J. (1953). *Anat. Rec.* **117**, 685.
Rapson, W. H. & Morbey, G. K. (1959). *T.A.P.P.I.* **42**, 125.
Saeman, J. F., Moore, W. E., Mitchell, R. L. & Millett, M. A. (1954). *T.A.P.P.I.* **37**, 336.
Schön, W. J. (1955). *Flora, Jena*, **142**, 347.
Wigglesworth, G. (1928). *Mem. Manchr lit. phil. Soc.* **72**, 157.
Wilson, C. M. (1959). *Analyt. Chem.* **31**, 1199.
Wise, L. E. (1958). *T.A.P.P.I.* **41**, 14A.
Yamanouchi, S. (1913). *Bot. Gaz.* **55**, 74.

Biochem. J. (1960) **77**, 508

The Determination of Amino Sugars in the Presence of Amino Acids and Glucose

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The presence of other sugars and amino acids interferes with the method for quantitative estimation of glucosamine and galactosamine described by Elson & Morgan (1933). The procedure, which is based on the reaction of amino sugars with acetylacetone in an alkaline medium and subsequent development of a red colour by chromogens formed with *p*-dimethylaminobenzaldehyde, has been modified by several authors. Many of these modifications are quoted by Rondle & Morgan (1955) in their paper dealing with the improvement of the estimation. Immers & Vasseur (1952) have examined the interference arising from mixtures of amino acids together with glucose under various conditions. Schloss (1951) proposed a method which overcomes such interference by taking advantage of the fact that colour due to amino acids and glucose fades after a comparatively short time. In his study of the mechanism of the reaction Schloss (1951) stated that a number of chromogenic products are formed by heating hexosamines with acetylacetone in alkaline media; partial fractionation of the reaction mixture yielded one volatile liquid and two or more non-volatile solid chromogens. The use of a homogeneous fraction giving a colour with *p*-dimethylaminobenzaldehyde would seem safer than a complex mixture for an analytical procedure. One of us (Cessi, 1952) developed a method based on the separation of the volatile chromogen(s) for the estimation of amino sugars in strongly coloured solutions. The procedure has been now adapted to the quantitative estimation of amino sugars in the presence of amino acids and glucose.

EXPERIMENTAL

Materials and methods

Amino sugars. D-Glucosamine hydrochloride was obtained from Hofmann–La Roche and Co. (Basle, Switzerland) and recrystallized from water by addition of conc. hydrochloric acid. D-Galactosamine was a kind gift from Professor W. T. J. Morgan. Aqueous solutions were made from hydrochlorides, and weights are referred to free sugars.

Acetylacetone reagent. Colourless, redistilled acetylacetone (b.p. 138–140°) was stored in the refrigerator and dissolved (1 ml.) in 100 ml. of 0·5N-sodium carbonate–sodium bicarbonate buffer containing 0·1M-sodium chloride. A buffer of pH 9·8, as adopted in the proposed method, was obtained by dissolving 23·02 g. of sodium carbonate, 2·76 g. of sodium bicarbonate and 5·84 g. of sodium chloride/l. of solution. The pH was checked after the addition of acetylacetone with a glass electrode standardized against 0·05M-sodium tetraborate and adjusted if necessary. The reagent

was stable for 1 day in the refrigerator. The pH was unchanged after boiling in stoppered, all-glass tubes.

p-Dimethylaminobenzaldehyde reagent. Recrystallized *p*-dimethylaminobenzaldehyde (80 mg.) was dissolved in 100 ml. of absolute ethanol containing 3·5 ml. of conc. HCl. The solution was almost colourless and could be stored in the refrigerator for several days.

Distillation apparatus. Distillations were performed in an all-glass apparatus assembled with a 100 ml. spherical flask with short neck and a 20 cm. condenser. The apparatus was heated directly over a gas burner, brought to boiling in about 1 min. and distilled at the rate of 1 ml./min.

Spectrophotometer. The extinctions of the solutions were measured with a Beckman DU spectrophotometer, with cells of 1 cm. light path. Wavelength calibration was checked with reference to a sodium source.

Procedure

Colour reaction. A portion (2 ml.) of solution containing 5–50 μg. of amino sugar, or mixtures of the amino acids listed and glucose (see Table 1) were mixed with 5·5 ml. of acetylacetone reagent, pH 9·8, in glass-stoppered tubes and heated in a boiling-water bath for 20 min. After cooling in cold water the reaction mixture and three washings of 2 ml. of water were transferred from each tube to the distillation apparatus. Portions (2 ml.) were distilled into 10 ml. volumetric flasks containing 8 ml. of *p*-dimethylaminobenzaldehyde reagent. The extinctions were determined 30 min. later at 545 mμ. For comparison the same quantities of amino sugars and of amino acids and glucose were tested by the procedure given by Rondle & Morgan (1955). Lysine yielded a strong colour with the latter method and very little after the distillation method, for which a quantity ten times as large was therefore taken.

Table 1. *Comparison of extinctions obtained by the present method and that of Rondle & Morgan* (1955)

Extinctions were measured for 1 m-mole of compound in 1 ml. of final coloured solution. The reactions were carried out with: amino sugars, 5–50 μg.; methylamine, 5 μmoles; choline, 20 μmoles; amino acids, 20 μmoles in the presence of 25 μmoles of glucose. Lysine was tested by taking 1–5 μmoles with the first method and 10–50 μmoles with the second, in the presence of 25 μmoles of glucose.

Compound	Method of Rondle & Morgan (1955) ($E_{530\ m\mu}$)	Present method ($E_{545\ m\mu}$)
D-Glucosamine	7200	6800
D-Galactosamine	7200	6600
Lysine	3700	4
Alanine	360	8
Histidine	320	7
Valine	280	8
Serine	260	8
Glycine	230	9
Cystine	220	3
Arginine	200	4
Phenylalanine	190	8
Glutamic acid	100	4
Leucine	100	5
Tyrosine	100	10
Aspartic acid	90	3
Hydroxyproline	60	26
Methionine	40	2
Tryptophan	30	4
Methylamine	2600	570
Choline	0	0

RESULTS

Influence of pH. The procedure described was tested at different pH values of the acetylacetone reagent. The colour intensities obtained were independent of pH between pH 9·5 and 10·0 (Fig. 1);

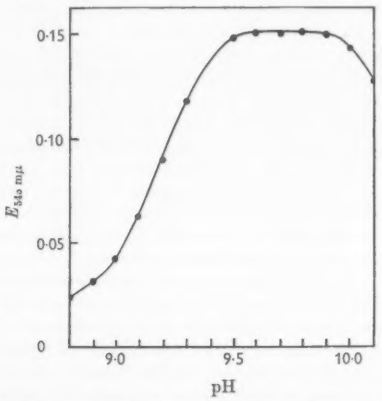

Fig. 1. Influence of pH during the reaction between amino sugars and acetylacetone on the intensity of colour. Each point was obtained with 40 μg. of D-glucosamine.

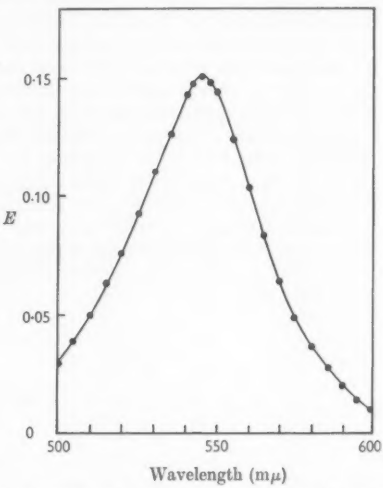

Fig. 2. Absorption spectrum of colour obtained by reaction with *p*-dimethylaminobenzaldehyde and chromogen(s) separated by distillation from the reaction mixture between 40 μg. of D-glucosamine and acetylacetone in alkali.

pH 9·8 has been chosen as standard value for all other experiments.

Absorption maximum. The absorption spectrum of the colour obtained with the described procedure showed a maximum at 545 mμ (Fig. 2). The colour was fully developed after 30 min. at room temperature and its intensity was unchanged after 24 hr.

Interference from amino acids and glucose. Table 1 gives a comparison of the extinctions of amino sugars and of amino acids in the presence of 25 μmoles of glucose with the procedure described and with the procedure of Rondle & Morgan (1955). With the former method lysine was found to give the most serious interference; alanine, valine and glycine interfered less. All interferences were practically completely overcome with the present method. The only seriously interfering substance was methylamine. Hydroxyproline was found to give a molar extinction about 0·3% of that of glucosamine. The addition of 0·02 M-sodium sulphite to the acetylacetone reagent decreased the colour due to hydroxyproline to one-tenth. Since the addition of sulphite reduces the sensitivity of the method it should not be added if hydroxyproline is known not to be present in large amounts. The method was tested with a hydrolysate of crystalline insulin that was known not to contain any amino sugar; 2 mg. of hydrolysate was used for the reaction. In one experiment 25 μmoles of glucose were added to the same quantity of hydrolysate. In no instance was colour observed.

Range, sensitivity and accuracy. The extinctions of the coloured solutions were found to be proportional to the amounts of amino sugars taken within the range 5–50 μg., and by decreasing the volume of the final solution it has been possible to estimate 2 μg. of hexosamine. The sensitivity could be increased by taking advantage of the concentration effected by distillation of the volatile chromogen(s), but this point has not been extensively investigated. The accuracy of the method was tested by determining the extinctions of 30 samples each containing 20 μg. of glucosamine, the determinations being carried out on three different days. The observed standard deviation was 2·4%.

DISCUSSION

The method described for the quantitative estimation of amino sugars uses the volatile fraction of chromogens formed by heating these compounds with acetylacetone in an alkaline medium. Among several substances investigated only methylamine was found to give interference. It is possible, however, that other volatile amines do so and their occurrence should be taken into account. The important interference by amino acids and glucose, relevant for lysine, is overcome with the present method. The procedure, which yields a stable colour, has been applied to a number of determinations of amino sugars in mucoproteins (C. Cessi, unpublished work; Johansen, Marshall & Neuberger, 1960).

SUMMARY

1. A method is described for the quantitative determination of amino sugars in the presence of amino acids and glucose. The procedure is based upon the observation of Schloss (1951) that amino sugars form volatile chromogen(s) when heated with acetylacetone in alkaline solution.

2. The volatile fraction is separated by distillation and a red colour is formed by reaction with *p*-dimethylaminobenzaldehyde.

3. The method overcomes the interferences due to amino acids in the presence of glucose, which form only non-volatile chromogens.

We are grateful to Professor G. Favilli, Dr C. Marsh and Dr R. L. M. Synge for their helpful suggestions and criticisms and to Professor W. T. J. Morgan for the gift of pure chondrosamine. We are indebted to G. Bellabarba for helpful technical assistance.

REFERENCES

Cessi, C. (1952). *Boll. Soc. ital. Biol. sper.* **28**, 858.
Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
Immers, J. & Vasseur, E. (1952). *Acta chem. scand.* **6**, 363.
Johansen, P. G., Marshall, R. D. & Neuberger, A. (1960). *Biochem. J.* **77**, 239.
Rondle, C. J. M. & Morgan, W. T. J. (1955). *Biochem. J.* **61**, 586.
Schloss, B. (1951). *Analyt. Chem.* **23**, 1321.

Metabolism of Bladder Carcinogens

3. THE METABOLIC PATH OF 2-[8-^{14}C]NAPHTHYLAMINE IN SEVERAL ANIMAL SPECIES*

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In previous papers (Henson, Somerville, Farquharson & Goldblatt, 1954; Somerville, Henson, Cooke, Farquharson & Goldblatt, 1956) we reported the results of investigations into the distribution of 2-[8-^{14}C]naphthylamine in the rat and rabbit, after intraperitoneal injection. We now present the results of similar studies on several different animal species, together with some observations on the absorption of the material into the blood stream through the skin and through the bladder wall.

MATERIALS AND METHODS

These were, with slight modification, as previously described.

Preparation of 2-amino-1-[8-^{14}C]naphthyl hydrogen sulphate. This was prepared by feeding 2-[8-^{14}C]naphthylamine (+inactive naphthylamine) to a dog and extracting the sulphuric ester from the urine. A portion (50 ml.) of the urine, adjusted to pH 4 with acetic acid, was passed through a column of charcoal deactivated with 4% stearic acid and the charcoal was washed with water. The adsorbed material was eluted with 100 ml. of saturated aq. phenol and 100 ml. of water and evaporated to dryness. It was repeatedly dissolved in water and evaporated to dryness and finally dried for several days *in vacuo* to remove the phenol. The brown, gummy residue was dissolved in butanol–ethanol–water (17:3:20, by vol.), made into a slurry with cellulose powder, and the slurry was placed on top of a dry cellulose powder column and eluted with the same solvent. The 2-amino-1-naphthyl hydrogen sulphate moves close behind the solvent front, so the first eluate fraction was removed and evaporated. The residue was boiled with ethanol, in which it was largely insoluble. Filtration and drying left about 90 mg. of a brown, powdery solid, which was shown by paper chromatography to consist mainly of the sulphuric ester; much smaller amounts of several slower-running materials were present but these were non-radioactive. The activity of the 2-amino-1-[8-^{14}C]naphthyl hydrogen sulphate [assayed as described by Henson *et al.* (1954) and Somerville *et al.* (1956)] was $4{\cdot}87 \times 10^4$ counts/min./mg.

Oxidation techniques. We have found exposure to hot reduced copper to be an unreliable method of removing oxides of nitrogen from CO_2 prepared from animal tissues for measurement in Geiger counters. Absorption by manganese dioxide (Belcher & Ingram, 1950; Christman, Day, Hansell & Anderson, 1955) has been found to give much more consistent results and to be a simpler method to apply.

RESULTS

Excretion of radioactivity from several animal species after intraperitoneal injections of 2-[8-^{14}C]-naphthylamine. The percentages of the dose excreted in the urine and faeces of dogs, guinea pigs and mice are shown in Table 1. Figures are given for excretion after 2 days (approx.) and for total excretion (after daily amounts had become negligible).

Table 1. *Percentage of dose excreted in urine and faeces of dogs, guinea pigs, mice, rabbits and rats after intraperitoneal injection of 2-[8-^{14}C]naphthylamine*

Doses: about 0·1 mg. to mice, 0·3–1·0 mg. to guinea pigs, rats and rabbits and 6 mg. to dogs.

	Percentage of dose excreted			
	Urine		Faeces	
Species	0–2 days	Total	0–2 days	Total
Dog	85·6	87·4	1·4	2·5
	74·0	75·4	3·0	3·2
Guinea pig	64·4	65·8	10·9	12·8
	78·4	79·9	11·0	14·2
	86·9	88·0	5·8	7·9
	79·2	80·0	5·4	6·4
Mouse	81·0	82·2	9·1	10·1
	77·2	78·4	10·7	12·8
	70·7	72·2	16·8	16·9
Rabbit*	81·7	—	7·8	—
	88·4	89·9	2·6	7·4
	81·8	82·4	12·5	13·3
Rat†	65·5	67·0	22·2	28·4
	60·9	61·8	26·5	28·1

* Values from Somerville, Henson, Cooke, Farquharson & Goldblatt (1956).

† Values from Henson, Somerville, Farquharson & Goldblatt (1954).

* Part 2. Somerville, Henson, Cooke, Farquharson & Goldblatt (1956).

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Chromatography of urine. Urines were collected for 24 hr. from a dog, rabbit, guinea pig, rat and mouse which had received intraperitoneal injection of 2-[8-^{14}C]naphthylamine, and were applied to Whatman 3MM paper, the volume for each animal being such that all spots contained about the same amount of radioactivity. One-dimensional chromatograms were run with two-solvent systems: butan-1-ol saturated with 2N-NH_3 soln., and butan-1-ol–ethanol–water (17:3:20, by vol.). The radioautographs of the chromatograms of the dog, rabbit, guinea pig, rat and mouse urines showed 1, 4, 6, 7 and 7 spots respectively and the most intense spots from dog, rat and mouse urine had the same R_F as did 2-amino-1-naphthyl sulphate.

Activity in blood and bile of a dog and a cat after intraperitoneal injection of 2-[8-^{14}C]*naphthylamine.* The bile ducts of a dog and of a cat were cannulated and bile was collected continuously after intraperitoneal injection of naphthylamine. Blood samples were taken periodically. The variation with time of the activity in blood and in bile are shown in Figs. 1 and 2; the patterns are similar to that for rabbits (Somerville *et al.* 1956, Fig. 3). The ratio of the concentration of activity in bile to that in blood, in the period after the peak level has been passed, varies from about 30 to 60 for the dog and 100 to 200 for the cat. Corresponding figures for the rabbit were 3–30 (Somerville *et al.* 1956) and for a rat 40–70. The total activity excreted in the bile of the dog

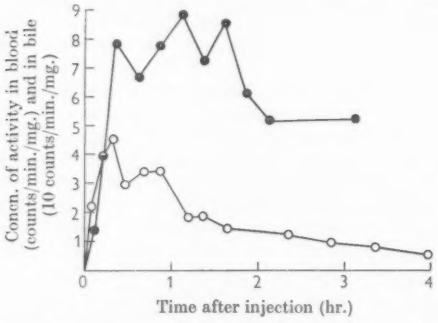

Fig. 1. Concentration of activity in blood and bile of dog 1 after intraperitoneal injection of 2-[8-^{14}C]naphthylamine. ○, Blood; ●, bile. Dose: $3{\cdot}20 \times 10^7$ counts/min.

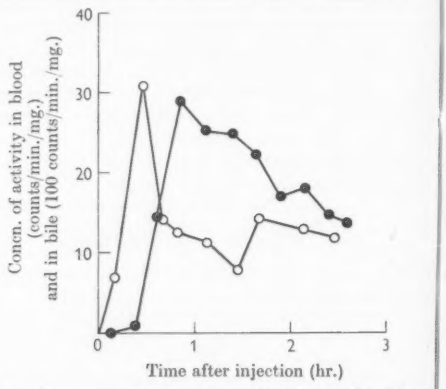

Fig. 2. Concentration of activity in blood and bile of cat 3 after intraperitoneal injection of 2-[8-^{14}C]naphthylamine. ○, Blood; ●, bile. Dose: $3{\cdot}39 \times 10^7$ counts/min.

Table 2. *Distribution of activity between blood plasma and cells in guinea pigs after intraperitoneal injection of* 2-[8-^{14}C]*naphthylamine*

Results are expressed as counts/min./mg. dry wt. Dose to each guinea pig: $4{\cdot}21 \times 10^7$ counts/min. (about 1·5 mg.). Animals weighed 600–700 g.

Time after injection (days)	Guinea pig 9		Guinea pig 10		Guinea pig 11		Guinea pig 12	
	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells
2	1·4	—	1·4	2·2	1·3	1·7	1·7	1·3
6	0	2·0	0·6	1·4	—	1·4	0·3	1·2
9	—	1·7	1·1	0·9	0·6	1·0	0	0·8
13	0	2·2	0	1·2	0	0·8	—	1·0
16	0·4	1·4	0	1·2	0	1·0	0	0·8
20	0·4	1·4	0·2	1·1	0·2	1·1	0·4	1·2
23	0	—	0	0·8	0	0·8	0	0·7
27	—	1·3	—	0·8	—	0·6	—	1·0
30	—	1·6	—	0·9	—	0·7	—	0·4
34	—	1·2	—	0·8	—	0·8	—	1·1
37	—	0·8	—	1·3	—	1·3	—	1·0
41	—	1·0	—	0·5	—	0·3	—	0·4
44	—	0·9	—	0·7	—	0·4	—	0·2
48	—	0·6	—	0·5	—	0·5	—	0·3
51	—	0·5	—	0·3	—	0·3	—	0·4
54	—	0·5	—	0·3	—	0·2	—	0·2
61	—	0·4	—	0	—	0·1	—	0·1
68	—	0·2	—	0·1	—	0	—	0·2

Table 3. *Distribution of activity between blood plasma and cells of a beagle dog after intraperitoneal injection of* 2-[8-^{14}C]*naphthylamine*

Wt. of dog, 10 kg. Dose: $2{\cdot}16 \times 10^8$ counts/min. (about 8 mg.). Results are expressed as counts/min./mg. dry wt. (Note: counting rates for samples from plasma after 22 days were only slightly above the minimum detectable level; accuracy of these results is therefore low.)

Time after injection (days)	Plasma	Cells
1	42·0	4·3
2	19·0	2·6
3	7·4	2·8
4	—	7·1
7	2·0	1·2
8	1·7	0·9
9	1·3	0·7
11	1·2	—
15	—	0·7
22	0·5	1·1
27	0·2	1·1
36	0·4	0·6
43	0·3	0·6
52	0·8	0·4

Table 4. *Distribution of activity between plasma, cell contents and cell membranes of rat blood after intraperitoneal injection of* 2-[8-^{14}C]*naphthylamine*

Blood of rat 45 was taken 13 days and of rat 46 20 days after injection. Dose to each rat: $1{\cdot}82 \times 10^7$ counts/min. (about 0·7 mg.). Results are expressed as counts/min. in the amount of blood constituent contained in 1 g. of whole blood.

	Rat 45	Rat 46
Plasma	37	22
Cell contents	3932	2900
Cell membranes	12	8

Table 5. *Activity in the blood of rat* 33 *after application of* 2-[8-^{14}C]*naphthylamine to the skin*

Dose: $3{\cdot}59 \times 10^7$ counts/min. (about 1·3 mg.). Results are expressed as counts/min./mg. wet wt.

Time (days) ...	1	2	3	6	7	8	9
Activity in blood	2·6	4·9	3·8	0·3	1·5	3·4	2·8
Time (days) ...	10	13	14	17	21	24	28
Activity in blood	2·9	2·1	2·0	1·7	1·4	1·1	0·7

in 3 hr. was 1·2 % of the dose and 2·2 % was found in the bile in the gall bladder. Corresponding figures for the cat are 12·2 and 0·03 % respectively in 2½ hr.

Partition of activity between blood constituents of dogs, guinea pigs and rats. Somerville *et al.* (1956) have shown that in rat blood the activity remains firmly attached to the cells after it has disappeared from the plasma, whereas in rabbits the activity disappears from the cells at about the same time as

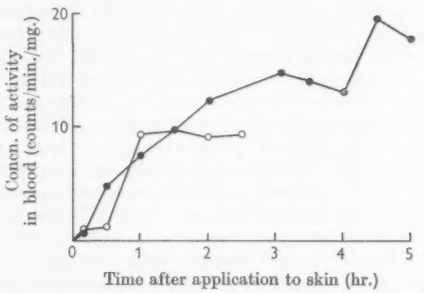

Fig. 3. Concentration of activity in blood of rats after application of 2-[8-^{14}C]naphthylamine to skin. ●, Rat 32; dose: $4{\cdot}54 \times 10^7$ counts/min. ○, Rat 33; dose: $3{\cdot}60 \times 10^7$ counts/min.

from the plasma. Results for guinea pigs and a dog are given in Tables 2 and 3. The site of the radioactive material which is firmly bound to rat-blood cells was further investigated by separating the cell contents from the membranes. Blood was taken from two rats at 13 and 20 days respectively after the intraperitoneal injection of naphthylamine. The cells were separated by centrifuging, washed twice with sodium citrate solution (22 g./l.) and then haemolysed with 6·7 mN-NH_3 soln. Cell contents and membranes were separated by centrifuging and the membranes washed five times with the NH_3 soln., the washings being added to the cell contents. Activity in the plasma, cell contents and cell membranes was measured (Table 4).

Activity in blood of rats and of a dog after application of 2-[8-^{14}C]*naphthylamine to the skin.* Absorption of naphthylamine through the skin has been studied in the rat. The animal's back was shaved and radioactive naphthylamine dissolved in a small volume of ethanol was applied. After the solvent had evaporated the area was covered with adhesive tape and the animal held in a restriction cage for the first few hours. Blood samples were taken from the tail and in every case appreciable amounts of activity were detectable in the blood after 10 min. The variation in the activity in the blood of rats 32 and 33 in the first few hours after application is shown in Fig. 3. In one case the activity in the blood was followed over a longer period; the results are given in Table 5. The fraction of the dose excreted in the urine of rat 32 was 10·8 % in 5 hr., and in rat 33 it was 14·8 % in 3 days.

A similar application was made to the skin of a dog (wt. 10 kg.). With a dose of about 6×10^7 counts/min. (2 mg.) definite evidence was obtained of penetration through the skin, although the counting rates of the samples were only just above the level of significance (3 counts/min.). Thus after

1 hr. a blood sample gave 5 counts/min. (0·1 count/min./mg. wet wt.) and the highest counting rate recorded was 12 counts/min. (0·08 count/min./mg. wet wt.) after 30 hr. (The highest concentration in the blood occurred a few hours after the skin application, but smaller samples were taken.) Amounts of this order were still detectable after 7 days.

Absorption of 2-[8-^{14}C]*naphthylamine and of* 2-*amino*-1-[8-^{14}C]*naphthyl hydrogen sulphate from the bladder into the blood of cats.* 2-[8-^{14}C]Naphthylamine (2·05 mg.) was suspended in approx. 0·05 M-KH_2PO_4 solution, dissolved with the minimum amount of HCl and the solution was brought to pH 3–4 with Na_2HPO_4. The total volume of 3 ml. was introduced by syringe and catheter into the bladder of an anaesthetized male cat, followed by 3 ml. of KH_2PO_4 solution, the bladder having previously been emptied of urine as thoroughly as possible after the introduction of the catheter. Blood samples were taken at intervals from the femoral vein. Concentrations of active material in the blood are shown in Table 6. If it is assumed that the total weight of blood was 5 % of that of the whole animal, the maximum concentration at 165 min. represents a fraction of about 3·3 % of the dose in the blood.

A similar experiment was performed with 2-amino-1-[8-^{14}C]naphthyl hydrogen sulphate, 54 mg. of which was dissolved in 4 ml. of KH_2PO_4 and Na_2HPO_4 solution (each approx. 0·05 M) adjusted to pH 7 with Na_2CO_3 and introduced by catheter into the bladder of an anaesthetized male cat, followed by 3 ml. of sodium chloride solution (8·5 g./l.). Blood samples were taken at intervals from the cannulated jugular vein, and concentrations of activity in the blood are shown in Table 7. On the same assumption as was made for cat 4, the maximum concentration at 220 min. represents a fraction of about 0·5 % of the dose in the blood.

Table 6. *Activity in the blood of cat* 4 *after introduction of* 2-[8-^{14}C]*naphthylamine into the bladder*

Dose: $5{\cdot}58 \times 10^7$ counts/min. (about 2 mg.). Wt. of cat, 2·5 kg. Results are expressed as counts/min./mg. wet wt.

Time after introduction (min.)	40	135	165	200	250
Activity in blood	8·0	14·0	14·5	12·8	9·0

Table 7. *Activity in the blood of cat* 5 *after introduction of* 2-*amino*-1-[8-^{14}C]*naphthyl hydrogen sulphate into the bladder*

Dose: $2{\cdot}63 \times 10^7$ counts/min. (54 mg.). Wt. of cat, 2·0 kg. Results are expressed as counts/min./mg. wet wt. (Counting rates were low. Each value is the mean from two samples each of about 150 mg.)

Time after introduction (min.)	40	70	100	130	160	190	220
Activity in blood	0·04	0·02	0·05	0·10	0·12	0·11	0·13

DISCUSSION

The excretion of naphthylamine or its metabolites, after intraperitoneal injection, takes a similar course in dogs, guinea pigs, mice, rabbits and rats in that almost the whole dose is excreted in 2 days (Table 1), but small amounts remain in the body and are detectable in the blood for several weeks afterwards [Tables 2 and 3 and Somerville *et al.* (1956), Tables 1 and 2]. Since this long-term retention of a small fraction of the dose has been shown to occur in four animal species it probably also occurs in man, and may have been significant in the induction of tumours in workers exposed to 2-naphthylamine, for their bodies would never have been entirely free of the material. The distribution of the excreted radioactivity between faeces and urine varies between species. The dog excretes only about 3 % of the dose in the faeces, the guinea pig, mouse and rabbit about 10 % and the rat almost 30 %. The small amount of activity in the bile of the dog with cannulated bile duct is concordant with the low excretion in the faeces. The fact that the dog excretes the highest fraction of the dose in the urine may be significant, in that the dog is the only experimental animal in which bladder tumours have been induced by feeding naphthylamine, but it cannot be of major importance, for even the rat excretes about two-thirds of the dose in the urine.

The experiments recorded in Tables 6 and 7 show that naphthylamine was absorbed from the bladder of a cat to an extent about seven times that of 2-amino-1-naphthyl hydrogen sulphate. Since the 'true' carcinogen (whether amine or metabolite), in order to exert its action on the proliferating layers of the bladder epithelium, must penetrate the mucous membrane at least as far as the submucous layer, it is evident that the amine can attain the site of carcinogenic action much more readily than can the sulphate. We suggest that it is possible that the carcinogenic action in man and dog might be due to small amounts of unchanged amine excreted in the urine, for Bonser, Bradshaw, Clayson & Jull (1956) have shown by the bladder-implantation technique that it is a carcinogen, if a weak one, and D. Manson (personal communication) has detected significant amounts of the base in the urine of dogs fed 0·2 g. of naphthylamine. In any case the ability to penetrate to the proliferating layers of cells is a prime requirement for any potential carcinogen and other metabolites which have been suggested as the 'true' carcinogen, e.g. 2-amino-1-naphthol, 2-amino-1-naphthyl glucosiduronic acid and bis-(2-

amino-1-naphthyl) phosphate, should be investigated from this point of view.

Naphthylamine or its metabolites appear very rapidly in the blood and bile of the dog and the cat (Figs. 1, 2) after intraperitoneal injection, their fate being very similar to that observed in rabbits (Somerville *et al.* 1956). The transfer to the bile from the blood is very rapid and the active transport across the barrier separating the blood and biliary systems is striking, particularly in the cat, where the concentration in the bile is more than a hundred times that in the blood.

The behaviour of the metabolites of naphthylamine in the blood of guinea pigs (Table 2) is similar to that in rats (Somerville *et al.* 1956), in that the active material is detectable in the blood cells long after it has disappeared from the plasma. There is the difference that the fraction of the dose found in the blood cells of the guinea pig within the first few days is much smaller than in the rat, and it does not decrease exponentially with time, but in a slower, more irregular manner. The results for the dog (Table 3) are probably comparable with those in the rabbit (Somerville *et al.* 1956), for, although the individual counting rates in samples of plasma were low in the later stages, it appears that the active material was present in the plasma as long as it was in the cells.

The results in Table 4 confirm the previous observations that, after intraperitoneal injection of active naphthylamine in the rat, there is considerable retention of activity in the blood cells after it has disappeared from the plasma, but, in addition, they show that, at 13 or 20 days, practically all the activity present in the blood was in the cell contents, not in the cell membranes. Since the average life of red cells in the rat is 45–50 days (Burwell, Brickley & Finch, 1953), the great majority of cells present at 13 or 20 days after injection would have been present at the time of injection; the data in Table 4 are therefore held to support the view that the activity in the cell contents is due to the passage of the active material across the cell membrane, with subsequent attachment to one or more of the constituents of the contents. This attachment must be strong to prevent the leaching out of the material into the plasma, or its removal by repeated washing of the cells in sodium citrate solution. Whatever the combination may be, it persists after the plasma and membrane activities have practically disappeared, which is in marked contrast with the behaviour of the liver carcinogen, 4-dimethylaminoazobenzene, which is also strongly bound to the blood cells in the rat but is mostly attached to the membranes (Zischka, Karrer, Hromotka & Broda, 1954). It might have been expected that, similarly, the naphthylamine would be attached to the lipid cell membrane, but it is bound to the cell contents. This may be evidence of the binding of a carcinogen to protein which is envisaged as the primary step in chemical carcinogenesis in the protein-deletion hypothesis (Miller & Miller, 1953).

In the clinical problem of occupational bladder tumours, the normal routes of absorption of the carcinogen have been variously held to be by the respiratory tract, by the skin or by both. Our findings recorded in Fig. 3 and Table 5 and the figures quoted for the dog show that both in the rat and the dog naphthylamine penetrates the skin in a short time. The concentration in the blood of the rats and the amounts found in their urine, in comparison with corresponding figures for animals which received naphthylamine by intraperitoneal injection, indicate that at least one-fifth of the dose applied to the skin was absorbed. It is more difficult to estimate the fraction absorbed by the dog, but it is probably of the order of a tenth. If human skin is penetrated with equal ease these quantities are of considerable significance in relation to the intense exposures suffered by workers in their employment 20 or more years ago.

SUMMARY

1. After the intraperitoneal injection of single doses of 2-[8-^{14}C]naphthylamine into the dog, rabbit, guinea pig, rat and mouse (approx. 1 mg./kg. body wt.) the dog excretes only about 3 % of the radioactive material in the faeces, the guinea pig, rabbit and mouse about 10–15 % and the rat almost 30 %. The amount in urine varied from 62 to 90 %.

The small amount excreted by the dog in the faeces is accounted for by low excretion in the bile.

2. Paper chromatograms of urines show the differences in metabolism of 2-[8-^{14}C]naphthylamine in these species.

3. The concentrations of radioactivity in the bile and in the blood of a dog and of a cat, after intraperitoneal injection, vary in a similar manner. The concentration at a given time is very much higher in the bile than in the blood.

4. Radioactivity is detectable in the blood of dogs and guinea pigs for several weeks after a single injection. In the dog the activity is shared between plasma and cells, but in the guinea pig after 3 weeks the activity is entirely in the cells.

5. The persistent activity in the blood of rats, after a single injection, is entirely in the contents of the cells, not in the plasma (previously shown) or in the red-cell envelope.

6. 2-[8-^{14}C]Naphthylamine is rapidly absorbed through the skin of the rat and the dog, being readily recognizable in the blood within a short time after application to the skin.

7. 2-[8-^{14}C]Naphthylamine is absorbed from the bladder into the blood of a cat to a notable extent, but 2-amino-1-[8-^{14}C]naphthyl hydrogen sulphate is absorbed very much less readily.

We wish to thank Dr D. Manson, Institute of Cancer Research, Pollard's Wood, for advice on the extraction of the metabolic 2-amino-1-naphthyl hydrogen sulphate from dog urine. We are indebted to Mrs M. E. Ross, Miss P. A. Melhuish, Mrs R. Coombes and Mr R. G. Harrison for technical assistance.

REFERENCES

Belcher, R. & Ingram, C. (1950). *Analyt. chim. acta*, **4**, 118, 401.

Bonser, G. M., Bradshaw, L., Clayson, D. B. & Jull, J. W. (1956). *Brit. J. Cancer*, **10**, 539.

Burwell, E. L., Brickley, B. A. & Finch, C. A. (1953). *Amer. J. Physiol.* **172**, 718.

Christman, D. R., Day, N. E., Hansell, P. R. & Anderson, R. C. (1955). *Analyt. Chem.* **27**, 1935.

Henson, A. F., Somerville, A. R., Farquharson, M. E. & Goldblatt, M. W. (1954). *Biochem. J.* **58**, 383.

Miller, J. A. & Miller, E. C. (1953). In *Advances in Cancer Research*, vol. 1, p. 388, Ed. by Greenstein, J. P. & Haddow, A. New York: Academic Press Inc.

Somerville, A. R., Henson, A. F., Cooke, M. E., Farquharson, M. E. & Goldblatt, M. W. (1956). *Biochem. J.* **63**, 290.

Zischka, W., Karrer, K., Hromotka, O. & Broda, E. (1954). *Mh. Chem.* **85**, 856.

Biochem. J. (1960) **77**, 516

A Graphical Method for the Rapid Determination of Sedimentation Coefficients

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The calculation of sedimentation coefficients by the usual methods, though not particularly difficult, can be time-consuming, particularly if the systems examined in the analytical ultracentrifuge are complex. Various aids to this calculation, such as tabulated values of the various constants required, are in general use, but these save only a small amount of the mathematics required. The procedure described in this paper completely eliminates all calculation, and also serves as a convenient means for recording the data. In addition, the sedimentation coefficients obtained are based on an assessment of all the data available for each run. A system which can achieve this degree of simplification requires preparation, but it is hoped that this paper will enable this to be done with the least possible effort on the part of the user.

PRINCIPLE OF THE METHOD

The determination of a sedimentation coefficient (S) depends upon the solution of the equation

$$S = \frac{\ln (x_2/x_1)}{\omega^2(t_2-t_1)}, \qquad (1)$$

where x is the distance of a boundary from the axis of a rotor rotating with an angular velocity of ω at time t (Svedberg & Pedersen, 1940).

Equation (1) shows that a graph of $\ln x$ against t, for a substance having a constant sedimentation coefficient, is a straight line having a slope which is a function of ω^2 and S. Consequently, if x is plotted on logarithmic graph paper, S may be read off this curve by means of a protractor calibrated in terms of rev./min. against S.

Both the graph paper and the protractors have been designed for use with the Spinco Model E ultracentrifuge (Beckman/Spinco Division, Palo Alto, Calif., U.S.A.), but there is no reason why similar apparatus should not be employed with other ultracentrifuges. This type of ultracentrifuge has a number of fixed speeds and fixed intervals between photographic exposures, and the number of possible combinations is very large, so that a certain amount of selection has had to be made. The speeds covered are from 21 740 to 59 780 rev./min. and times from 4 to 246 min., which covers adequately all S values from 1 to 250s, and it is possible to extend this range considerably, as is explained below.

APPARATUS

The graph paper. Graph paper covering the range required is not available commercially at present, but it may be prepared fairly simply by one of several methods. It is conveniently included as part of a special data sheet, which

serves as a record of the run in question. The scale (Fig. 1) has as an abscissa time-intervals, eight in all, spaced 2 cm. apart. The ordinate is calibrated logarithmically in terms of x, from $x = 5{\cdot}9$ to $x = 7{\cdot}1$ by using a scale of 0·01 log units = 2 cm. (Table 1).

The ratio of the two scales is, of course, the important factor, but the dimensions given are convenient in use. This calibrated area (Fig. 1), which is approx. 16·1 cm. × 14 cm., may be printed on a sheet of duplicating paper 21 cm. × 33 cm. in size, which can contain the following information in addition: material under investigation, with concentration and composition of solvent; date; speed of centrifuge; exposure interval and time; revolution-counter readings; drive voltage; photographic material; solvent viscosity and density; corrected S; comments. Schlieren angles are recorded with exposure number, and the uncorrected S values are recorded beside the appropriate lines on the graph.

The graph paper may be made by drawing on a Durotype stencil No. 6 (Gestetner Ltd., London) with pen No. SP2 and a Gestetner writing sheet. Alternatively, it may be drawn in Indian ink on a sheet of paper and duplicated on a stencil by the Gestefax (Gestetner Ltd.) process, which is a dry electronic stencilling process and gives stable stencils. Copying by ordinary photographic processes is not sufficiently reliable because of the uneven stretching of the paper.

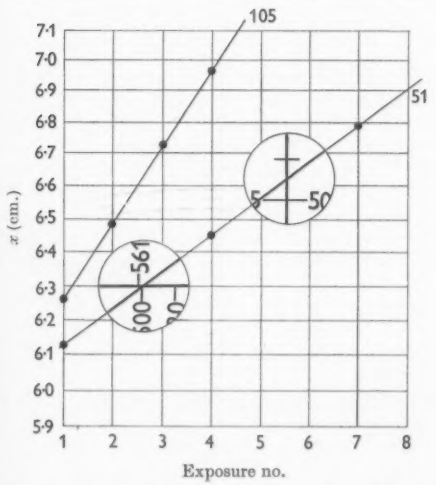

Fig. 1. The logarithmic graph paper, showing enlarged, in the circles, the use of the 4 and 16 min. protractor (Fig. 2). On the left, the lower protractor scale is fitted at 35 600 rev./min. to the intersection of one of the horizontal graph lines (6·3 cm.) and the experimental curve. On the right, the experimental curve is seen to intersect the protractor ordinate scale at $S = 51$ s. The data are taken from a run (4/1/60) made at 35 600 rev./min. and 21·1°, with 4 min. exposure intervals, of turnip yellow mosaic virus (upper curve: $S = 105$ s) and the nucleic acid-free protein (lower curve) in 0·1 M-KCl. Total protein concentration approx. 5 mg./ml.

Table 1. *Ordinate values for the logarithmic graph paper with abscissa intervals of 2 cm.*

x (cm.)	Ordinate (cm.)
7·1	16·08
7·0	14·85
6·9	13·60
6·8	12·33
6·7	11·04
6·6	9·72
6·5	8·41
6·4	7·06
6·3	5·68
6·2	4·31
6·1	2·88
6·0	1·46
5·9	0·00

Table 2. *Abscissa distances for 4 and 16 min. protractor, in units of* s *on the 4 min. ordinate*

A convenient size is given by 150 s = 100 mm. The 16 min. ordinate scale is one-tenth that of the 4 min. ordinate scale, and the abscissa figures have been adjusted to take this into account.

Rev./min.	4 min.	16 min.
21 740	184·7	—
23 150	163·0	—
24 630	144·0	—
26 980	129·5	—
27 690	113·9	—
29 500	100·4	—
31 410	88·5	—
33 450	78·1	—
35 600	69·0	172·4
37 020	63·8	159·5
39 460	56·1	140·3
42 040	49·5	123·6
44 770	—	109·0
47 660	—	96·2
50 740	—	84·8
52 640	—	78·8
56 100	—	69·4
59 780	—	61·1

Table 3. *Abscissa distances for 8 and 32 min. protractor, in units of* s *on the 8 min. ordinate*

The 32 min. ordinate is one-tenth the scale of the 8 min. ordinate. In order to make the two protractors of approximately equal size, a scale of 60 s = 6 in. is recommended. In this case, the figures in the table will be in 0·1 in. units.

Rev./min.	8 min.	32 min.
29 500	50·22	—
31 410	44·27	—
33 450	39·05	—
35 600	34·50	—
37 020	31·90	—
39 460	28·06	—
42 040	24·73	—
44 770	21·80	—
47 660	19·24	48·1
50 740	16·96	42·4
52 640	15·77	39·4
56 100	13·88	34·7
59 780	12·23	30·6

The protractors. These are calculated from equation (1) by using values for the intervals between exposures of 4, 8, 16 and 32 min. For 4 min. exposures the abscissa distance to the left of the ordinate may be calculated for any centrifuge speed from the relationship

$$y = 8{\cdot}738 \times 10^{10}/(\text{rev./min.})^2 \text{ Svedberg units (s)}, \quad (2)$$

and the other scales are in proportion to the time intervals used.

A considerable simplification of the construction of the protractors may be effected by using two ordinate scales in the proportion of 10:1, so that only one set of ordinate calibrations is necessary. This has been done in both pro-

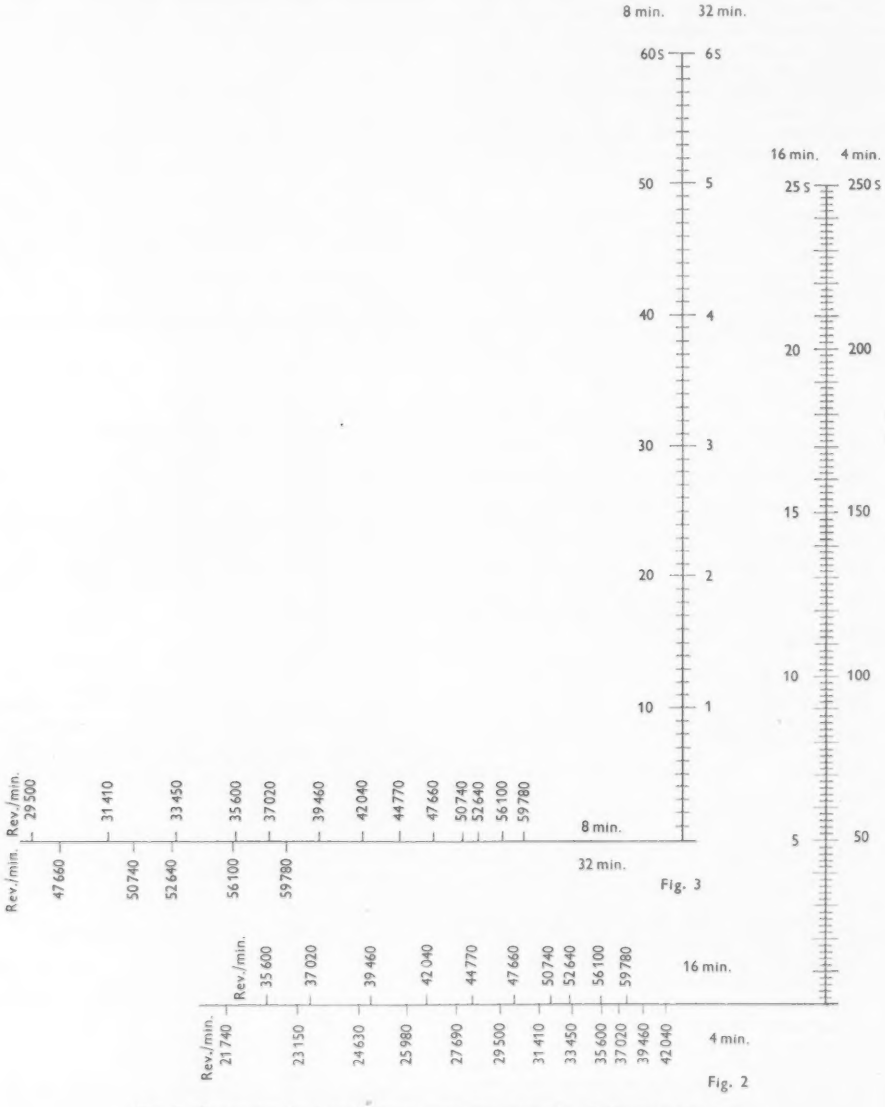

Fig. 2. Protractor for 4 and 16 min. exposure intervals. Ordinate scales in s.

Fig. 3. Protractor for 8 and 32 min. exposure intervals. Ordinate scales in s.

tractors in combination with a fourfold difference in the time intervals used. The abscissa scales are then in the proportion of 2·5:1. These protractors are illustrated in Figs. 2 and 3. The data used in making them are given in Tables 2 and 3, which also include an indication of a convenient size for each protractor, though this may be varied to suit any particular requirement.

The actual protractor scales illustrated cover the range of s values most commonly encountered, and they may be extended by a number of devices. For example, the 4 min. scale, if used with 2 min. exposure intervals in the ultracentrifuge, will give half the correct S value, and so S values as high as 500s may be determined. Furthermore, the low gear-speeds from 3397 to 9341 rev./min. (numbered in red) may be used in conjunction with the corresponding normal speeds (numbered in white), when the S value determined from the protractor needs to be multiplied by 36 (the square of the gear-box ratio). This extends the range of values which may be measured to 9000s.

It is important in preparing the scales that a stable material should be used. Ideally they should be drawn on card at least twice the size required, and reduced photographically onto a process-type cut film (Kodaline KS3, Kodak Ltd., London). Both protractors (Figs. 2, 3) can be incorporated onto the same sheet of film. The most important consideration in preparing the protractors is that the ordinate and abscissa scales should be exactly at right angles to each other.

HOW TO USE THE METHOD

The experimental values of x are read off from the ultracentrifuge plates in the usual way, and plotted on the logarithmic graph paper (Fig. 1). The appropriate protractor (Fig. 2 or 3) is then placed on the graph with its abscissa scale along a horizontal graph line (in Fig. 1, left, this is on line $x = 6{\cdot}3$ cm.), with the line corresponding to the rotor speed at the intersection of the experimental curve with the latter. The experimental curve will then intersect the ordinate scale of the protractor at the required value of S (Fig. 1, right).

The objective of the system described above was first and foremost convenience. It is quite obvious that the problem may be solved by using simpler equipment, e.g. by making the protractor scales integral with the graph paper, and using parallel scales to transfer the slopes, but it was thought that this method was not quite so convenient in use.

As far as accuracy is concerned, it has been found in practice that results are reliable within about ± 1s in 100s if boundary positions are estimated to within 0·01 cm. The step involving the plotting of log x against t is particularly valuable because it serves as a check that sedimentation is progressing uniformly, besides ensuring that the value for S is based on a weighted assessment of all the experimental observations. If two or more components are present during a run, it is possible to check that the two curves intersect at the level of the meniscus; and, if a peak is just resolved at the end of a run, it is possible to draw a line from its position to the meniscus, and so estimate the slope accurately.

SUMMARY

1. A graphical method for the evaluation of sedimentation coefficients is described. Its principle involves the employment of a special logarithmic graph paper and protractors calibrated directly in Svedberg units.

I should like to thank Mr S. Frey for the trouble which he has taken with the photographic problems involved.

REFERENCE

Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge*, p. 17. Oxford University Press.

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The Reactions of some Aromatic *C*-Nitroso Compounds with Haemoglobin

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Although the reactions of many ligands with methaemoglobin have been described, work with haemoglobin has, until recently, been confined to the low molecular weight molecules of oxygen, carbon monoxide and nitric oxide. St George & Pauling (1951) have, however, examined the equilibrium between haemoglobin and a series of *iso*-cyanides, finding a sharp decrease in affinity on passing from *iso*propyl *iso*cyanide to *tert.*-butyl *iso*-cyanide. With free haem, on the other hand, they found much smaller differences in affinity. To explain their results, they put forward the idea that the haem groups of haemoglobin are located in a 'crevice' in the protein which hinders the approach

of the bulky *tert.*-butyl *iso*cyanide molecule, but allows easier access to the smaller *iso*propyl *iso*cyanide molecule. Recently, Ainsworth, Gibson & Roughton (1960) have given an account of kinetic measurements with the series of alkyl *iso*cyanides, showing that the difference in affinity of the several members is wholly accounted for by differences in their rates of combination with reduced haemoglobin; this result is exactly contrary to the position for oxygen, carbon monoxide and nitric oxide where the million-fold difference in affinity depends chiefly on differences in the rate of dissociation.

It has been known for nearly 40 years that nitrosobenzene will combine with haemoglobin, and Jung (1940) has shown that in nitrosobenzene–haemoglobin the ratio nitrosobenzene to iron is 1:1 and that nitrosobenzene and carbon monoxide compete with one another for combination. The nitrosobenzene molecule, with its bulky aromatic nucleus and the facility of introducing substituents with known relations to the active centre, offers an opportunity to extend the observations already made with the *iso*cyanides to a further group of compounds, and this paper describes the results obtained with nitrosobenzene and the nitrosotoluenes.

EXPERIMENTAL

Blood solutions. Stock solutions of sheep reduced haemoglobin, of undetermined electrophoretic type and corresponding to blood diluted 1:4, were prepared exactly as described by Gibson & Roughton (1957*a*), and were stored in a refrigerator for up to a week. Working solutions were prepared by diluting the stock solution with a 2% (w/v) soln. of $Na_2B_4O_7,10H_2O$, and all determinations were made with this borate buffer solution. Crystalline whale myoglobin was a gift from Professor S. R. Elsden.

Nitroso compounds. Nitrosobenzene was prepared according to Vogel (1948), *o*-nitrosotoluene by the method of Bamberger & Rising (1901*a*), and *m*- and *p*-nitrosotoluenes as described by Bamberger & Rising (1901*b*). In each case the corresponding hydroxylamine was separated from the reaction mixture and crystallized from light petroleum before oxidation to the nitroso compound. All the melting points were within 1·5° of those given in the references quoted. The nitroso compounds were stored at −12°, and appeared to be stable for several months at that temperature.

Solutions were prepared by shaking an excess of nitroso compound with the 2% soln. of borate warmed to about 50°. The mixture was then cooled, kept for 10 min. and excess of nitroso compound was filtered off. The concentration of solutions prepared in this way was determined by dilution with water (1:50 was usually convenient) and measurement of the extinction at 320 mμ in 1 cm. cells. Conversion of the readings into concentrations of nitroso compounds was done by comparison with a standard curve obtained by dissolving weighed amounts (about 10–15 mg.) in 50 ml. of ethanol and then diluting with water. Dilution to 1:50 with water gave a reading at 320 mμ of about 0·4. The solutions of nitroso compounds do not seem to be very stable and were always prepared immediately before use.

Stopped-flow determinations. These were made with the apparatus and procedure of Gibson (1954). The combination reactions with haemoglobin were followed at 435 mμ and the replacement of nitroso compounds by CO was followed at 600 mμ.

Concentrations of haemoglobin solutions. These were determined spectrophotometrically with a Unicam SP. 600 at 576 mμ, taking the millimolar extinction coefficient of O_2Hb as 15·6. All concentrations are stated in terms of haemin, not in molarity of haemoglobin of molecular weight 68 000.

Notation. The combination of nitroso compounds with haemoglobin, like that of other ligands, is believed to take place in four stages as suggested by Adair (1925). Following Antonini & Gibson (1960) the individual velocity constants are written as lower-case letters with two subscript numbers the first of which is the number of ligand molecules bound before reaction, and the second the number after. Thus the first step in the combination reaction is written h_{01}, and the first dissociation reaction is h_{43}. The letter h is used to distinguish the nature of the ligand, k and l being used for O_2 and CO respectively. The four equilibrium constants are written as capital italic letters, the subscript giving the number of ligand molecules combined in the more fully saturated of the two compounds concerned. Thus the equilibrium constant of the first reaction between reduced haemoglobin and a nitroso compound is written H_1.

RESULTS

Combination of reduced haemoglobin with nitroso compounds. The rate constant for this reaction was determined by mixing together, in the stopped-flow apparatus, reduced haemoglobin and an oxygen-free solution of nitrosobenzene dissolved in the buffer used for the haemoglobin. When the reaction was followed with several different concentrations of nitrosobenzene the initial value for the rate constant was greater the more dilute the nitrosobenzene solution (Fig. 1). It is well known [see, for example, the recent review of Gowenlock & Lüttke (1958) for references] that aromatic nitroso compounds readily form unreactive dimers both in solution and in the solid state. If the dimers do not react with haemoglobin, dependence of the apparent rate constant on the concentration of nitroso compound would be expected. The curve in Fig. 1 has been calculated assuming a monomer–dimer equilibrium, and agrees well with the experimental points. The value of the rate constant for the combination of haemoglobin with each of the compounds studied was obtained by carrying out an experiment similar to that shown in Fig. 1 and extrapolating to infinite dilution of nitroso compound.

The values are collected in Table 1. This shows that the rates are all similar except for *o*-nitrosotoluene which combines only about one-fifth as rapidly as the other compounds.

Course of the reaction between nitrosobenzene and haemoglobin. Plotting the logarithm of the ratio of

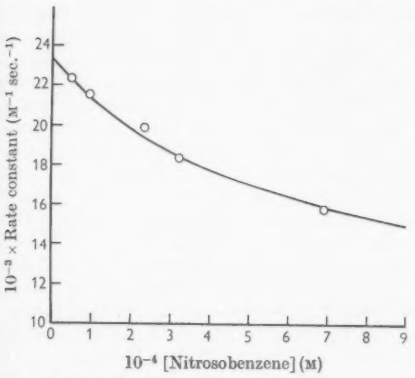

Fig. 1. Combination of reduced haemoglobin with nitrosobenzene. The figure shows the effect on the second-order rate constant of varying the concentration of nitrosobenzene. The concentration of haemoglobin was 30 μM in borate buffer, pH 9·1, The observations were made at 20° with light of mean wavelength 435 mμ with a depth of solution of 2·34 mm. The curve was calculated on the assumption that the variation in rate is due to an equilibrium in the solution between dimeric and monomeric forms of nitrosobenzene with dissociation constant $2{\cdot}2 \times 10^{-3}$ M. The ordinate is the overall rate constant, equal to 0·25 h_{01}.

Table 1. *Combination of nitroso compounds with haemoglobin and myoglobin*

The figures give the rate of combination extrapolated to zero concentration of ligand. The figure for haemoglobin is h_{01}, giving the rate of combination of the first molecule of ligand. Borate buffer, pH 9·1; temp. 20°. Myoglobin: observations at 437 mμ, 2·34 mm. light path; concn. 19 μM after mixing. Nitroso compounds: concn. 0·1–0·5 mM.

Compound	Rate of combination: Myoglobin ($10^{-4} \times M^{-1}$ sec.$^{-1}$)	Haemoglobin ($10^{-4} \times M^{-1}$ sec.$^{-1}$)
Nitrosobenzene	9·2	9·2
o-Nitrosotoluene	8·0	1·7
m-Nitrosotoluene	10·8	10·5
p-Nitrosotoluene	10·0	10·5

the concentration of reactants in a second-order reaction against time should give a straight line. When this procedure was applied to the combination of haemoglobin with nitrosobenzene a gently rising curve was obtained (Fig. 2, line *A*), a result reminiscent of Gibson & Roughton's (1955) finding for the combination of haemoglobin with carbon monoxide. They attributed the deviation from the second-order equation to differences in the rate with which successive molecules of carbon monoxide combined with a single molecule of haemoglobin, and later gave an account of methods by which the four velocity constants for the successive reactions

$$Hb_4 + CO \rightarrow Hb_4CO,$$
$$Hb_4CO + CO \rightarrow Hb_4(CO)_2,$$
$$Hb_4(CO)_2 + CO \rightarrow Hb_4(CO)_3,$$
$$Hb_4(CO)_3 + CO \rightarrow Hb_4(CO)_4$$

could be determined (Gibson & Roughton, 1957*a*). Applying their methods, values of the first constant h_{01} were obtained from the initial slope of plots similar to that in Fig. 2, and h_{34} was taken to be $1{\cdot}3 \times 10^5$ M^{-1} sec.$^{-1}$, as determined independently by methods to be described later. Values of the two remaining constants h_{12} and h_{23} were selected by

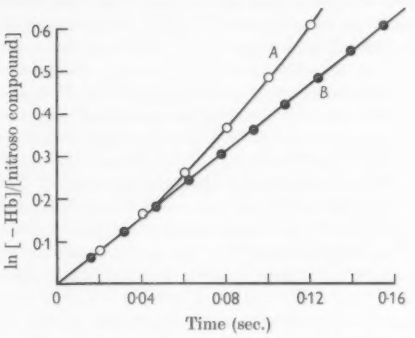

Fig. 2. Plot showing that *o*-nitrosotoluene reacts with reduced haemoglobin in an accurately second-order way (*B*), whereas the reaction with nitrosobenzene speeds up as it progresses (*A*). The units of the ordinate are arbitrary and have been adjusted to make the initial slopes coincide for the two compounds. The reactions were followed with light of mean wavelength 435 mμ, with 30 μM-haemoglobin dissolved in borate buffer pH 9·1, at a temperature of 20°.

Table 2. *Comparison of the course of the reaction between nitrosobenzene and reduced haemoglobin as observed and as calculated*

The calculated values were obtained by Desk Method II of Gibson & Roughton (1957*a*), taking the ratios of the velocity constants for the combination of successive molecules of nitrosobenzene to be 4:3:2·5:5·5. The experimental values were obtained with the stopped-flow apparatus with 1·03 mM-nitrosobenzene and 50 μM-haemoglobin at 20·3° and pH 9·1. Concentrations given are those before mixing.

Time (msec.) ...	20	40	60	80	100	120
Nitrosobenzene–haemoglobin observed (%)	16·0	31·5	45·1	57·9	67·5	75·8
Nitrosobenzene–haemoglobin calculated (%)	16·7	31·6	45·1	57·0	67·4	75·5

trial and error, and the calculated and observed courses of the reaction compared with Desk Method II of Gibson & Roughton (1957*a*). An example of the results is given in Table 2, which shows that a reasonable fit was obtained with $h_{01}:h_{12}:h_{23}$ as 4:3:2·5. These ratios do not differ significantly from those (4:6:2) found by Gibson & Roughton (1957*a*) for sheep haemoglobin combining with carbon monoxide, though they are closer to the ratios (4:3:2) which would be obtained if there were no haem–haem interaction until three molecules of nitrosobenzene had combined.

Course of the reaction between haemoglobin and the nitrosotoluenes. When the results with *m*- and *p*-nitrosotoluenes are compared with those for nitrosobenzene, it is found that the curves for all three are superimposable, and the ratios of the combination velocity constants must therefore be similar. This is not true of *o*-nitrosotoluene, however, where the reaction appears to be accurately second-order throughout its course (Fig. 2, line *B*). This difference between *o*-nitrosotoluene and the other compounds examined has been confirmed in experiments with different samples of blood and in each case the result of Fig. 2 has been reproduced. There are two main ways of explaining this result: either there is no haem–haem interaction with *o*-nitrosotoluene, when values for successive velocity constants would be in the ratio 4:3:2:1, or haem–haem interaction takes place between pairs of haems and gives rise to ratios of, say, 4:10:2:10, where the exact numerical value of the second and fourth constants is not important, provided only that they are both substantially larger than the first and third velocity constants. The choice between these alternatives is considered in the Discussion.

Effect of temperature on the combination of haemoglobin with nitrosobenzene. Determinations of the rate of combination, at three temperatures and two concentrations of nitrosobenzene, were made. The natural logarithms of the rate constants are shown plotted in the usual way against the reciprocal of absolute temperature in Fig. 3. It is clear that the 6·4-fold difference in concentration of nitrosobenzene did not influence the temperature dependence of the reaction so that the slope of the lines in Fig. 3 is a measure of the activation energy of the nitrosobenzene–haemoglobin reaction, and not of the effect of temperature on, for example, the monomer–dimer equilibrium of nitrosobenzene. The separate lines for the two concentrations of nitrosobenzene are due to the dependence of the observed rate constant on concentration shown in Fig. 1. The value of the activation energy derived from the slope of the lines in Fig. 3 is 13·8 kcal.

Combination of myoglobin with nitroso compounds. The rates of combination with reduced myoglobin

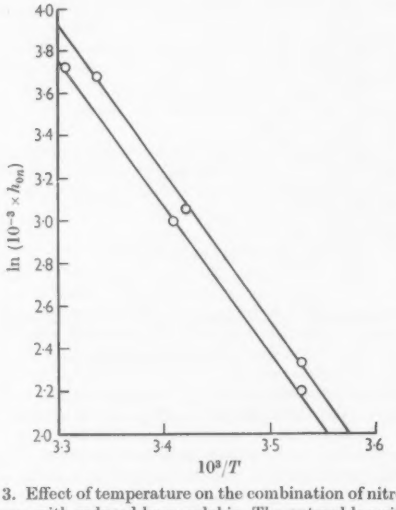

Fig. 3. Effect of temperature on the combination of nitrosobenzene with reduced haemoglobin. The natural logarithm of the overall rate constant (= 0·25 h_{01}) is plotted against $1000/T$, where T is the absolute temperature of the experiments. The lower line was obtained with 0·59 mM-nitrosobenzene, the upper line with 92 μM. The concentration of haemoglobin was 31 μM in borate buffer, pH 9·1. The reaction was followed with light of mean wavelength 435 mμ.

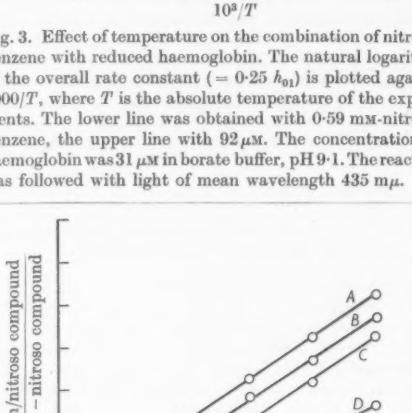

Fig. 4. Combination of myoglobin with nitrosobenzene and the nitrosotoluenes. The logarithm of the ratio of myoglobin remaining to nitroso compound remaining, divided by the difference in concentration between the myoglobin and the nitroso compound, is plotted against time. The slope of the lines is proportional to the second-order velocity constant. The ordinate is arbitrary. The concentration of myoglobin, determined as metmyoglobin cyanide, was 19 μM, the temperature was 20°, and the reaction was followed with light of mean wavelength 437 mμ. *A*, Nitrosobenzene, 66 μM; *B*, *p*-nitrosotoluene, 131 μM; *C*, *m*-nitrosotoluene, 112 μM; *D*, *o*-nitrosotoluene, 86 μM.

are given in Table 1 and are similar to those for haemoglobin (with the exception that the rate of combination of *o*-nitrosotoluene is much nearer to that for the other compounds than with haemoglobin). The course of the reaction is accurately second-order in every case, and examples for the four compounds are given in Fig. 4. This result shows that changes in the concentration of the monomer, which might be expected to follow on mixture of the solution of nitroso compound with an equal volume of myoglobin solution, are either much faster or much slower than the combination reactions with myoglobin. This point is important in justifying the kinetic analysis of the reactions with haemoglobin where the complex nature of the reaction might obscure changes due to dissociation of the nitroso compounds.

Dissociation velocity constants. The rate of dissociation of the first molecule of nitroso compound from fully saturated haemoglobin has been determined by displacement with carbon monoxide. The principles of the method have been fully discussed by Gibson & Roughton (1957*b*); the ratio l_{34}/h_{34} is sufficiently high to allow direct determinations of h_{43} in just the same way as nitric oxide can be used to determine l_{43} (Gibson & Roughton, 1957*b*) without the extrapolation procedure of Gibson & Roughton (1955) [l_{34} is the velocity constant for the reaction $Hb_4(CO)_3 + CO \rightarrow Hb_4(CO)_4$, and l_{43} that for the reverse reaction]. The results, given in Table 3 show that nitrosobenzene and *m*-nitrosotoluene dissociate at about the same low rate, which is about half that for carbon monoxide (Gibson & Roughton, 1957*b*). *o*-Nitrosotoluene dissociates about twice as fast as nitrosobenzene, whereas *p*-nitrosotoluene dissociates about 12 times as fast. The temperature coefficient for the dissociation of nitrosobenzene is 4·1/10°, which is similar to that for carbon monoxide.

Relative affinity of haemoglobin for oxygen and nitroso compounds. Where the numbers of free ligand-binding groups are negligible, Roughton (1934) has shown that the partition of haemoglobin between two ligands is given by [HbY][X] = [HbX][Y] × partition constant, where X and Y are the two ligands. The partition constant is itself equal to the ratio of the equilibrium constants for the combination of the fourth molecules of the two ligands. In the case of carbon monoxide and oxygen this relation is $L_4/K_4 = M$, where L_4 is the equilibrium constant for the reaction $Hb_4(CO)_3 + CO \rightleftharpoons Hb_4(CO)_4$ and K_4 that for the corresponding reaction with oxygen. Exactly analogous relations apply to any pair of ligands which do not react with one another, and, as the value of K_4 is known, the equilibrium constant for the combination of the fourth molecule of a nitroso compound may be obtained by examining its competition with oxygen.

In practice, a solution of oxyhaemoglobin in buffer had a measured amount of nitroso compound added, and was then equilibrated with a known partial pressure of oxygen. The proportions of oxyhaemoglobin and of nitrosohaemoglobin were then determined spectrophotometrically, and this allowed H_4, the equilibrium constant for the combination of the fourth molecule of nitroso compound to be calculated.

The equation [HbY][X] = [HbX][Y] × constant, may, if the total concentration of haemoglobin is taken as 1, be re-written log [HbY/(1−HbY)] = log constant + log Y − log X; if the concentration of X is kept constant, a plot of log [HbY/(1−HbY)] against log Y should give a straight line of unit slope. Fig. 4 shows such plots for the four nitroso compounds examined; the values of H_4 derived from them are given in Table 3.

Velocity of combination of the fourth molecule of the nitroso compounds with haemoglobin. By combining the data for H_4 and h_{43} given in Table 3, values of h_{34}, the combination velocity constant for the fourth molecule of nitroso compound to combine with haemoglobin, may be obtained. These values are given in Table 3, together with the ratio $4h_{34}/h_{01}$, which ranges only between 5 and 9 and has an average value of 7. This average value is to be compared with the statistical value of 1 which would be expected in the case that no haem–haem interaction existed.

Table 3. *Rates of combination and dissociation of the fourth molecule of nitroso compounds reacting with haemoglobin*

h_{43} was determined by CO displacement: blood diluted/100 in borate buffer, pH 9·1, in the form of nitrosohaemoglobin was mixed with mM-CO in borate buffer, 20°; observations were made at 600 mμ. H_4 was determined by competition between nitroso compounds and O_2: blood diluted/100 in borate buffer, in equilibrium with air (1 atm. pressure), was mixed with varied quantities of nitroso compounds, shaken with air and the extinction was read at 600 mμ; final concentration of haemoglobin, 38 μM.

Compound	h_{43} (sec.$^{-1}$)	$10^{-5} \times H_4$ (M^{-1})	$10^{-4} \times h_{34}$ (M^{-1} sec.$^{-1}$)	$4h_{34}/h_{01}$
Nitrosobenzene	0·016	80	13	5·5
o-Nitrosotoluene	0·032	9·5	3·0	7·1
m-Nitrosotoluene	0·021	108	23	8·8
p-Nitrosotoluene	0·20	7·0	14	5·3

DISCUSSION

The experiments have been made more laborious and less certain by the tendency of the nitroso compounds to dimerize in aqueous solution. No changes in extinction were, however, observed when making spectrophotometric measurements of the concentration of solutions, nor was a wait necessary when making dilutions of the stock nitroso compound solutions in carrying out experiments of the kind shown in Fig. 1. If there were a rapidly changing monomer–dimer equilibrium during the combination reactions, the calculation of second-order rate constants would be disturbed in experiments similar to those shown in Figure 1, because, as the combination reaction proceeded, the dimer initially present would decrease, owing to the taking up of part of the monomer by the haemoglobin. This effect may be rendered unimportant either by measuring initial rates, or by working with a considerable excess of nitroso compound, and one or other of these practices has been followed throughout.

One other source of difficulty requires discussion. Jackson & Thompson (1954) have shown that iodonitrosobenzene and iodophenylhydroxylamine can combine with haemoglobin at sites other than those occupied by ligands such as oxygen and carbon monoxide. They find, for example, a stoicheiometric uptake of the radioactive iodo compounds by carboxyhaemoglobin, unaccompanied by spectral changes. No evidence of interference, due to combination reactions of this kind, has been found in the experiments described here. It is not possible to say with certainty if this is because the conditions used here were unsuitable for the combination or because the tests available were not adequate to detect it. The experiments on the competition of oxygen with nitrosobenzene and *m*-nitrosotoluene offer the best chance of detecting combination with groupings other than the haem group. In these experiments a small measured quantity of nitroso compound was mixed with a known amount of haemoglobin. The quantity of free nitroso compound in equilibrium with the oxyhaemoglobin was calculated from the amount of nitrosohaemoglobin formed and the amount of nitroso compound added. If, besides combining with the haem group, some of the nitroso compound had combined with another group in the haemoglobin molecule, the calculation of the amount of free nitroso compound would be inaccurate, and this inaccuracy would be reflected in plots of the kind given in Fig. 5. The effect to be expected depends on the relative affinity of the haem group and the second site. In the limiting case where the affinity of the two groups is widely different, if the haem group has the lower affinity, no combination will be observed until one equivalent of nitroso compound has been added; in less extreme cases the plots would be concave upwards; Fig. 5 shows no tendency of this kind, suggesting that the affinity of the second site may be lower than that of the haem group. Such a suggestion is entirely compatible with Jackson & Thompson's (1954) findings, since in their example they used 25 mg. of iodophenylhydroxylamine in 10·5 ml., giving an initial concentration of about 10 mM, compared with the concentration of about 20 μM required in the experiments of Fig. 5 for half-displacement of oxygen.

It would also be possible for effects, due to the combination of further molecules of nitroso compounds, to show up in the course of the combination reaction with reduced haemoglobin and myoglobin. As the reaction with haemoglobin is kinetically complex, no conclusions can be drawn. With myoglobin, no side-effects were observed, the course of the combination being accurately second-order throughout. There does not seem to be any reason for thinking that reactions of the kind described by Jackson & Thompson (1954) have interfered measurably with the results given here.

The results for the compounds examined show surprising variations in the rates of the reactions with haemoglobin, whereas with myoglobin the rate is about the same with all four compounds (Table 1). With haemoglobin, the rate of combination of both the first and fourth molecules of *o*-nitrosotoluene is about one-fifth of that for the other three compounds, and both rates differ widely from that for myoglobin. With the other three nitroso compounds the numerical values of h_{01}, h_{34} and h_{0n} (for myoglobin) all agree within a factor of

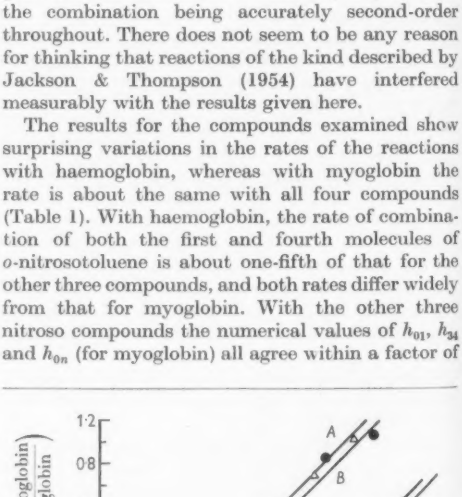

Fig. 5. Competition between oxygen and nitroso compounds for combination with haemoglobin. The total concentration of haemoglobin was 0·8 mM, in borate buffer, pH 9·1 at 20°. Determinations of the concentration of nitrosohaemoglobin were made spectrophotometrically at 600 mμ. *A*, *m*-Nitrosotoluene; *B*, nitrosobenzene; *C*, *o*-nitrosotoluene; *D*, *p*-nitrosotoluene.

two, and the agreement between h_{01} and h_{0n} is almost exact. This result is very different from that reported by Ainsworth *et al.* (1960) for a series of alkyl *iso*cyanides (ethyl, *iso*propyl, *tert.*-butyl) where the rate of combination with myoglobin correlated well with the rate of combination of the fourth molecule with haemoglobin, but showed poor correlation with the rate of combination of the first molecule of *iso*cyanide. In this respect the nitroso compounds resemble the gaseous ligands more closely than the *iso*cyanides.

It is surprising that *p*-nitrosotoluene should show a rate of dissociation about ten times as high as that for the other three compounds: just as *o*-nitrosotoluene forms an exception so far as the rates of combination are concerned, so *p*-nitrosotoluene is exceptional in its rate of dissociation.

The behaviour of *o*-nitrosotoluene offers the further puzzle that the course of the combination reaction with haemoglobin is accurately second order. As the $4h_{34}/h_{01}$ ratio is 7 (Table 3) the possibility that there is no haem–haem interaction is excluded, since this would require that $4h_{34}/h_{01} = 1$. The alternatives which remain are that h_{12} and h_{23} are both considerably greater than h_{01} [in such a case the extrapolation procedure of Gibson & Roughton (1957*a*), would yield high values for h_{01}] or that interaction occurs between pairs of haems, i.e. $h_{12} \gg h_{01}$ and $h_{34} \gg h_{23}$. The data do not permit a choice to be made between these possibilities, but interaction between pairs of haems is the more likely.

The haem–haem interaction, as measured by the ratio of the first and last velocity constants and given in Table 3, is about the same for all four compounds and gives the average value 7. In the *iso*cyanide series already mentioned, the comparable ratio varies from 2·3 for ethyl *iso*cyanide to 78 for *tert.*-butyl *iso*cyanide (Ainsworth *et al.* 1960), whereas for the gaseous ligands the values are CO = 60, O_2 = 30, NO = 4 (Gibson, 1959). The value for the nitroso compounds is thus similar to that for nitric oxide. It is tempting to take the series of values for haem–haem interaction as a whole and try to account for them by a single hypothesis. For the gaseous ligands, whose molecules occupy much the same volume, but which show wide fluctuations in interaction, the size of the interaction varies inversely with the absolute rate of combination of the first ligand molecule. Such an effect would be expected if, as a result of haem–haem interaction, the rate of combination of the fourth ligand molecule reached a maximum largely independent of the chemical nature of the ligand molecule. This idea can readily be extended to the *iso*cyanides where the absolute value of the velocity constant for the combination of the fourth molecule of *iso*cyanide decreases only tenfold on passing from ethyl to *tert.*-butyl *iso*cyanide, whereas the rate of combination of the first molecule decreases 500-fold in the same series. The nitroso compounds examined do not show large variations in haem–haem interaction, but the average of 7 is within the range for the *iso*cyanide series. Consideration of the absolute rates for the combination of the fourth ligand molecule is, however, less satisfactory, for the rate for the nitroso compounds is considerably larger than that for the *iso*cyanides, which must presumably be put down to the chemical differences between them.

The recent striking success of Perutz *et al.* (1960) and of Kendrew *et al.* (1960) in constructing models showing the relation of the haem groups to the polypeptide chains for haemoglobin and myoglobin lends additional interest to the discussion of haem–haem interaction, for the models show that the structures of oxyhaemoglobin and oxymyoglobin are closely similar. The question then arises whether reduced myoglobin more nearly parallels, in its behaviour, fully reduced haemoglobin or haemoglobin combined with three molecules of ligand. The evidence on this point is not yet complete and appears contradictory. If attention is given only to the rates of reaction, the gaseous ligands and the nitroso compounds react with myoglobin as though it were one-quarter of fully reduced haemoglobin, whereas the *iso*cyanides behave as though myoglobin corresponded to haemoglobin in which three places were already occupied by ligand molecules. Further work, including particularly the determination of the activation energies of the reactions, is required to clear up this point.

The rate of combination of the first molecule of nitrosobenzene and the activation energy lead to a value of the frequency factor similar to that found for other ligands (with the notable exception of oxygen combining with haemoglobin). A summary of the results and a figure has been given by Gibson (1959). The *iso*cyanides have been found by Ainsworth *et al.* (1960) to give a similar value for the frequency factor as does the rate of dissociation of the first molecule of nitrosobenzene from fully saturated haemoglobin. Such data are difficult to interpret unambiguously, but it seems that there is no special difficulty about the approach of the nitrosobenzene molecules to within reaction radius of the haem group. Certainly the compounds examined here are well below the limiting size for approach to the haem group, for Kiese & Plattig (1958) have shown that haemoglobin is able to react both with benzylphenylhydroxylamine and the corresponding nitroso compound. Detailed kinetic studies with haemoglobin would be difficult because of the insolubility of these compounds in water.

SUMMARY

1. Reduced haemoglobin combines successively with four molecules of nitrosobenzene and the nitrosotoluenes.

2. The rate constants for the combination of the first molecule of ligand at pH 9·1 and 20° are (M^{-1} sec.$^{-1}$): nitrosobenzene, $9{\cdot}2 \times 10^4$; *o*-nitrosotoluene, $1{\cdot}7 \times 10^4$; *m*-nitrosotoluene, $10{\cdot}5 \times 10^4$; *p*-nitrosotoluene, $10{\cdot}5 \times 10^4$.

3. The fourth molecule to combine does so more rapidly than the first, the average increase in rate is sevenfold. The increase compared with the first molecule is about the same for all four compounds.

4. The rate of dissociation of the first molecule of the same compounds from fully saturated haemoglobin has been measured. For nitrosobenzene, and *o*- and *m*-nitrosotoluene, the rates lie within the range 0·016–0·032 sec.$^{-1}$ at 20°. The value for *p*-nitrosotoluene is 0·2 sec.$^{-1}$.

5. The activation energies for the combination of the first molecule of nitrosobenzene and the dissociation of the first molecule of nitrosobenzene give a normal value to the frequency factor in both cases.

6. The rates of combination of the four nitroso compounds with myoglobin are numerically equal to those for the combination of the first molecule with reduced haemoglobin, with the exception of *o*-nitrosotoluene which combines with myoglobin almost as fast as do the other compounds.

REFERENCES

Adair, G. S. (1925). *J. biol. Chem.* **63**, 529.
Ainsworth, S., Gibson, Q. H. & Roughton, F. J. W. (1960). *Proc. Roy. Soc.* B, **152**, 331.
Antonini, E. & Gibson, Q. H. (1960). *Biochem. J.* **76**, 534.
Bamberger, E. & Rising, A. (1901*a*). *Liebigs Ann.* **316**, 279.
Bamberger, E. & Rising, A. (1901*b*). *Liebigs Ann.* **316**, 284.
Gibson, Q. H. (1954). *Disc. Faraday Soc.* **17**, 137.
Gibson, Q. H. (1959). *Progr. Biophys.* **9**, 1.
Gibson, Q. H. & Roughton, F. J. W. (1955). *Proc. Roy. Soc.* B, **143**, 310.
Gibson, Q. H. & Roughton, F. J. W. (1957*a*). *Proc. Roy. Soc.* B, **146**, 206.
Gibson, Q. H. & Roughton, F. J. W. (1957*b*). *Proc. Roy. Soc.* B, **147**, 44.
Gowenlock, B. G. & Lüttke, W. (1958). *Quart. Rev. chem. Soc., Lond.*, **12**, 321.
Jackson, H. & Thompson, R. (1954). *Biochem. J.* **57**, 619.
Jung, F. (1940). *Biochem. Z.* **305**, 248.
Kendrew, J. C., Dickerson, R. E., Strandberg, B. E., Hart, R. G., Davies, D. R., Phillips, D. C. & Shore, V. C. (1960). *Nature, Lond.*, **185**, 422.
Kiese, M. & Plattig, K. H. (1958). *Arch. exp. Path. Pharmak.* **233**, 484.
Perutz, M. F., Rossmann, M. G., Cullis, A. F., Muirhead, H., Will, G. & North, A. C. T. (1960). *Nature, Lond.*, **185**, 416.
Roughton, F. J. W. (1934). *Proc. Roy. Soc.* B, **115**, 451.
St George, R. C. C. & Pauling, L. (1951). *Science*, **114**, 629.
Vogel, A. I. (1948). *Practical Organic Chemistry*, p. 603. London: Longmans Green and Co. Ltd.

Biochem. J. (1960) **77**, 526

The Estimation of Phospholipase A Activity in Aqueous Systems

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The action of snake-venom phospholipase A on phospholipid fractions was first investigated in aqueous reaction systems. Levene & Rolf (1923) and Levene, Rolf & Simms (1924) carried out the reaction in phosphate buffer, which has been used extensively since that time (Chargaff & Cohen, 1939; Fairbairn, 1945; Rimon & Shapiro, 1959). In phosphate buffer, however, the conversion of lecithin into lysolecithin by venom preparations is slow and incomplete. For example, Chargaff & Cohen (1939) reported a lysolecithin yield of from 15 to 50% of the original lecithin employed in a reaction system maintained at 38° for a period of 24 hr.

The finding that phospholipase A can split lecithin in ether solution (Hanahan, 1952; Hanahan, Rodbell & Turner, 1954) has been of great practical importance. The ethereal reaction system was further studied and improved by Long & Penny (1957), and is used generally at the present time for the preparation of lysolecithin. The system is somewhat inflexible, however, as some diacyl phospholipids are relatively insoluble in ether, and pH adjustment is difficult.

The rate at which phospholipase A attacks lecithin or other phospholipids has been followed by a

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variety of different analytical methods. The release of free fatty acid during the reaction has been determined both by direct titration (Fairbairn, 1945; Hanahan, 1952; Hanahan *et al.* 1954) and by manometric techniques (Zamecnik, Brewster & Lipmann, 1947; Habermann, 1957). Long & Penny (1957), Rapport & Franzl (1957) and Rimon & Shapiro (1959) followed the course of lecithin hydrolysis by measuring the decrease in acyl ester bonds taking place. This is a relatively simple procedure, is very reproducible and permits the use of less substrate in the assay system than other methods.

The present work was undertaken in an attempt to devise an aqueous medium which would be suitable for the determination of phospholipase A activity in crude enzyme preparations or tissue homogenates. A 2:4:6-collidine-buffered system containing ether is described in which the hydrolysis of ovolecithin by phospholipase A goes rapidly to completion. Enzyme activity was measured by the decrease in acyl ester bonds during the reaction. The system has been used to investigate some of the properties of snake-venom phospholipase A. A modification of the basic method is described which permits its use with commercial Pancreatin or other crude tissue preparations.

EXPERIMENTAL

Materials

Ovolecithin. Ovolecithin was prepared from fresh egg yolks, and was purified by chromatography on an alumina column according to the method of Rhodes & Lea (1957). It was stored in brown glass bottles at $-15°$ in chloroform solution. For certain experiments, the ovolecithin was washed by the procedure described by Folch, Lees & Sloane-Stanley (1957) to remove Ca^{2+} ions.

Sources of phospholipase A. The dried venom of the cottonmouth moccasin (*Agkistrodon piscivorus piscivorus*) was purchased from Ross Allen's Reptile Institute, Silver Springs, Florida, U.S.A. Pancreatin was obtained from British Drug Houses Ltd.

Buffers. Phosphate buffer (0·067 M) was prepared from KH_2PO_4 and Na_2HPO_4 stock solutions. 2-Hydroxymethylpropane-1:3-diol (tris)–maleate buffer (0·05 M) was made up as described by Gomori (1955). The preparations of diethyl barbiturate (veronal)–HCl (0·04 M), triethanolamine–HCl (0·05 M) and 2:4:6-collidine–HCl (0·05 M) buffers are described by Dawson & Elliott (1959). Final pH adjustment was made by using a glass electrode. Triethanolamine and 2:4:6-collidine were redistilled before use. Other chemicals were commercial samples of the purest grade available.

Methods

Measurement of enzyme activity. Phospholipase A activity was measured by following the decrease in acyl ester bonds occurring during the conversion of lecithin into lysolecithin. Ester content was determined by a hydroxamic acid method, essentially as described by Stern & Shapiro (1953). The hydroxylamine reagent was prepared as required by dissolving 2·09 g. of recrystallized hydroxylamine hydrochloride in 15 ml. of water and adding 15 ml. of 3·5 N-NaOH.

Some difficulty was experienced in the selection of a suitable standard for the ester determination, as different substances produced slightly different colour yields/acyl ester bond. It was finally decided to use the ovolecithin itself as a standard. This was made possible by the fact that both ovolecithin and lysolecithin produced coloured solutions obeying Beer's law over a concentration range of 0·13–2·00 μg./sample. The colour yield of lysolecithin/μmole was half that of ovolecithin. Lysolecithin was not attacked by the phospholipase A, or any other enzyme, in the reaction systems described.

The extinctions of individual samples were calculated as a percentage of the extinction of the zero-time controls. The values thus obtained were used to calculate the amount of hydrolysis (%) that had taken place, and hence the amount (μg.) of ovolecithin hydrolysed. A correction was applied to compensate for the lysolecithin content of the original substrate.

Snake-venom reaction system. An appropriate portion of the ovolecithin stock solution was dried *in vacuo*. Buffer was added and the lipid emulsified by vigorous shaking before final volume adjustment. In most experiments, 1 ml. of lipid emulsion contained 1 mg. of ovolecithin. Samples (1 ml.) of the ovolecithin emulsion were pipetted into glass-stoppered test tubes (110 mm. × 17 mm.). The tubes were tightly stoppered after the addition of a quantity of diethyl ether (usually 0·1 ml.), and placed in a rack. The tubes were immersed in a water bath at 30°, and shaken continuously during temperature equilibration and the subsequent reaction period.

Immediately before use, an aqueous solution of the moccasin venom was prepared, and brought to a temperature of 30°. In a typical experiment, 2 mg. of dried venom were dissolved in 1 ml. of 5 mM-$CaCl_2$ solution. At fixed time intervals, 0·05 ml. of the venom solution was added to each reaction tube. After the appropriate reaction period, enzymic hydrolysis was stopped by the addition of 2 ml. of ethanol and 1 ml. of hydroxylamine reagent (ethanol alone did not stop the reaction immediately).

All samples were examined in duplicate. In the zero-time samples containing ovolecithin, the ethanol and hydroxylamine reagent were added before the enzyme. After the addition of the hydroxylamine reagent, the tubes were left on the bench for a minimum of 20 min.

Colour development was carried out by the addition of 0·6 ml. of 3·3 N-HCl, followed, after thorough mixing, by the further addition of 0·5 ml. of $FeCl_3$ reagent (Stern & Shapiro, 1953). The samples were read against a reagent blank (zero-time, no ovolecithin) in a spectrophotometer at a wavelength of 525 mμ.

In a few instances it was noticed that a slight turbidity or outright precipitate appeared in the sample after the addition of the $FeCl_3$ reagent. This sometimes occurred when the substrate concentration was high. It has also been observed during the analysis of high concentrations of lysolecithin and other phospholipids, in the presence of excessive free fatty acid, and with certain other enzyme sources. The addition of 1 ml. of diethyl ether to such samples (including all others in the set) cleared them completely.

Pancreatin reaction system. To each test tube was added 0·5 ml. of ovolecithin emulsion (2 mg. of ovolecithin/ml. of

buffer), 0·5 ml. of pancreatin syspension (50 mg. of pancreatin/ml. of buffer), 0·05 ml. of 5 mM-$CaCl_2$ solution, and 0·1 ml. of ether. The tubes were tightly stoppered and maintained at 30° with constant shaking as above. The reaction was stopped by adding 4 ml. of chloroform–methanol (2:1, v/v) to each tube and shaking vigorously for 30 sec. After centrifuging, the upper aqueous phase and the solid material at the boundary between the two phases were aspirated off. A 2 ml. sample of the lower phase was transferred to another glass-stoppered test tube. The solvents were carefully evaporated off under reduced pressure. The tubes were dipped periodically into a bath of hot water during the evaporation procedure. Once the solvents had been removed, each tube had added to it 3 ml. of 67% aq. ethanol and 1 ml. of hydroxylamine reagent.

All samples were prepared in duplicate, and colour development was carried out as described above.

RESULTS

In preliminary experiments, ovolecithin was emulsified in a variety of different buffers, and calcium-containing moccasin-venom solutions were added. Although the nature of the buffering agent made some difference to the yield of lysolecithin, hydrolysis was always incomplete. In an experiment with phosphate buffer (0·067 M, pH 6·5) 68% of the ovolecithin was hydrolysed after 2 hr.; contact with the enzyme for a further period of 18 hr. had no additional effect. This result is in agreement with the findings of other authors. For example, Fairbairn (1945) calculated a yield of fatty acids from a mixed lipid substrate that was 72% of the theoretical, in a reaction carried out in a phosphate-buffered (0·05 M, pH 7·0) medium with moccasin venom as the source of phospholipase A.

The addition of ether to buffered reaction media increased both the rate at which lecithin was converted into lysolecithin and the final amount of lysolecithin produced. The nature of the buffering agent used in such an ether-containing system had a considerable influence on the rate of the reaction. Fig. 1 shows that the hydrolysis of ovolecithin by moccasin-venom phospholipase A was relatively slow in phosphate, succinate, malonate, tris–maleate and triethanolamine buffers, more rapid in diethyl barbiturate buffer, and most rapid in 2:4:6-collidine buffer. The time courses of hydrolysis in phosphate, succinate, malonate and tris–maleate buffers were very similar, and are represented in the figure by mean values only. The curve for 2:4:6-collidine buffer was plotted with mean values obtained in eight experiments, and the standard errors of the means are indicated. All the buffers were of approximately the same molar strength; the pH was adjusted to 6·5 in them all.

The nature of the effect of the buffering agent on the reaction rate has not been further investigated.

The beneficial effect of 2:4:6-collidine when it was used as a buffering agent does not appear to be due to an effect similar to that produced by ether, as is shown in the next section

Effect of ether. The effect of the addition of various volumes of diethyl ether to the collidine-buffered reaction system is shown in Fig. 2. Without added ether the reaction proceeds slowly and only 16% of the substrate was hydrolysed at the end of a 1 hr. reaction period. The maximum rate of hydrolysis was attained with 0·1 ml. of ether in the system, an amount almost sufficient to saturate the buffer.

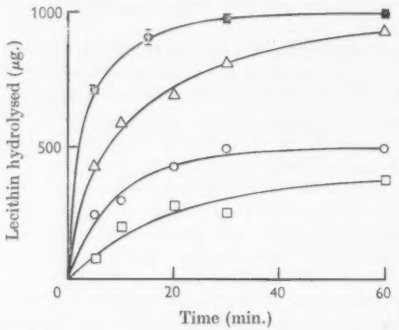

Fig. 1. Time course of hydrolysis of ovolecithin by moccasin-venom phospholipase A in different buffers at pH 6·5. Reaction mixtures contained: 1 mg. of ovolecithin in 1 ml. of buffer, 0·1 ml. of ether and 100 μg. of venom added in 0·05 ml. of 5 mM-$CaCl_2$. Temp. 30°. ●, 2:4:6-Collidine buffer, 0·05 M (mean ±S.E.M. for eight experiments); △, diethyl barbiturate buffer, 0·04 M; ○, triethanolamine buffer, 0·05 M; □, mean values obtained with phosphate (0·067 M), succinate (0·05 M), malonate (0·05 M) and tris-maleate (0·05 M) buffer.

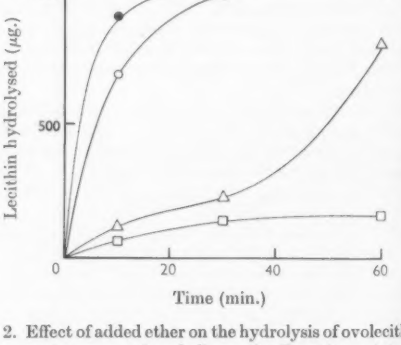

Fig. 2. Effect of added ether on the hydrolysis of ovolecithin by moccasin-venom phospholipase A. Reaction mixtures contained: 1 mg. of ovolecithin in 1 ml. of 2:4:6-collidine buffer (0·05 M, pH 6·5), 100 μg. of venom was added in 0·05 ml. of 5 mM-$CaCl_2$. □, No added ether; ○, with 0·05 ml. of ether; ●, with 0·10 ml. of ether; △, with 1·0 ml. of ether. Temp. 30°.

With 1·0 ml. of added ether the reaction velocity was initially low, but became more rapid after about 30 min. This was a biphasic system, with a layer of ether above the buffer. The peculiar nature of the curve can be explained largely on the basis of pH, as in such biphasic systems the ether gradually extracted the 2:4:6-collidine from the underlying buffer. The result was that the buffer became more acidic, finally stabilizing at about pH 5·5. Curves, similar to the one shown with 1·0 ml. of ether and 100 μg. of venom in the system, have been observed in experiments carried out in buffers at pH values slightly below 6. In an experiment with only 10 μg. of venom/sample, it was observed that hydrolysis in the biphasic system during the initial 30 min. reaction period was less than, but similar to, that in the curve shown (Fig. 2). However, during the 30–60 min. time period the hydrolysis stopped completely. This phenomenon also occurs in experiments conducted at pH values below 6 with 10 μg. of venom/sample.

The distribution of ovolecithin, venom and Ca^{2+} ions in a biphasic system is undoubtedly very complicated. Hanahan (1952) found that lecithin was extracted from buffer by ether in the form of a complex with the enzyme, in which the Ca^{2+} ions may also be bound (Long & Penny, 1957). The venom itself is not ether-soluble (Hanahan *et al.* 1954).

Some attempts were made to replace the ether with other reagents, all of which, however, interfered with the ester linkage determination. Deoxycholate, saponin and Tweens (Atlas Powder Co., U.S.A.) at a final concentration of 1 % in the buffer, or replacing the ether with dioxan gave extremely high blank readings. The addition of 0·1 ml. of either light petroleum (b.p. 40–60°) or 4-methylpentan-2-one to a suspension of ovolecithin (1 mg./ml.) in 2:4:6-collidine buffer decreased the final colour yield in the ester method by 25 %. 4-Methylpentan-2-one had earlier been found to be a good solvent in which to prepare lysolecithin from ovolecithin. Under comparable conditions the reaction velocity in the ketone was equal to that in ether; the reaction could be carried out at a temperature of 37°.

The mechanism by which ether, in appropriate concentration in the buffer, stimulates phospholipase A activity is obscure. It may be related, on an enzyme–substrate level, to the coalescence observed by Kates & Gorham (1957) of lecithin micelles and plant plastids in the presence of ether and certain other organic solvents.

Effect of pH. The time course of hydrolysis of ovolecithin by moccasin-venom lecithinase A at pH 6·0, 6·5 and 8·0 is shown in Fig. 3. At pH 6·0 there appeared to be a short lag period in the reaction between the 3 and 6 min. time intervals (observed in all experiments at this pH), after which the conversion of lecithin into lysolecithin progressed more rapidly. At pH 6·5 the reaction went smoothly to completion. The initial reaction velocity at pH 8·0 was greater than that at pH 6·5, but the rate of hydrolysis fell off more quickly, being only 85 % complete after 1 hr., with little or no change after a further 1 hr. incubation. At pH 6·5 there is no initial time lag in the hydrolysis, as there is in the ethereal reaction system (Long & Penny, 1957).

Fig. 4 shows that the initial reaction velocity was decreased at pH 5·5 and 6·0, but was almost constant from pH 6·5 to 8·5. This curve is very similar to that found with cholinesterase acting on acetylcholine (Laidler, 1955). It does not appear to agree with previous reports dealing with the effect of pH on snake-venom phospholipase A activity. Levene *et al.* (1924) stated that cobra venom was active between pH 6·5 and 7·5 and completely inactivated at pH 8·0. Hughes (1935) reported that the rate at which snake venoms attacked surface films of lecithin was optimum at pH 7·3. Hanahan (1952) suggested that the optimum range lay between

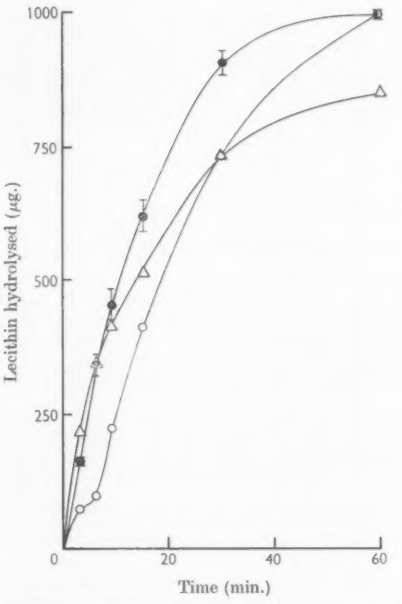

Fig. 3. Effect of pH on the time course of hydrolysis of ovolecithin by moccasin-venom phospholipase A. Reaction mixtures contained: 1 mg. of ovolecithin in 1 ml. of 2:4:6-collidine buffer (0·05 M), 0·1 ml. of ether and 10 μg. of venom added in 0·05 ml. of 5 mM-$CaCl_2$. Temp. 30°. ○, pH 6·0; ●, pH 6·5 (mean ±S.E.M. for eight experiments); △, pH 8·0.

pH 6·5 and 7·0. In all the previous work, however, initial reaction velocities were not determined and relatively long incubation periods were employed. Fig. 4 demonstrates that an apparent optimum at pH 6·5–7·0 is observed when the values obtained after a 30 min. reaction period are plotted. This is explained by the fact that the moccasin-venom phospholipase A is gradually degraded in solutions with a pH higher than 7·0, as is clearly shown in the curve for the preincubated enzyme preparations. Snake-venom phospholipase A is apparently quite stable in slightly acid solutions, but not in alkaline solution, confirming the early observations of Levene *et al.* (1924). Hughes (1935) reported that snake-venom phospholipase A is stable to prolonged boiling at pH 5·9, but is rapidly destroyed in solutions more alkaline than pH 7·0.

The high initial reaction velocities observed at the higher pH values are not the result of spontaneous hydrolysis of the substrate under these conditions. Emulsions of ovolecithin in 0·05 M-2:4:6-collidine buffer at pH 8·5 were quite stable during prolonged incubation at 30°.

Effect of venom and substrate concentrations. With increasing venom concentrations in the reaction system the initial reaction velocity increased linearly at first (Fig. 5), fell off rather abruptly at a venom concentration of between 25 and 50 μg./sample, and then decreased gradually up to the highest concentration used (100 μg./sample). The non-linearity of the rate with increasing venom concentration may be due to some physical limitation in the system, for example availability of substrate to enzyme. With the lowest concentration of venom studied (1·25 μg./sample), the reaction did not go to completion, even after prolonged incubation.

The effect of substrate concentration on the initial reaction velocity is shown in Fig. 6. When the mean experimental values shown were replotted

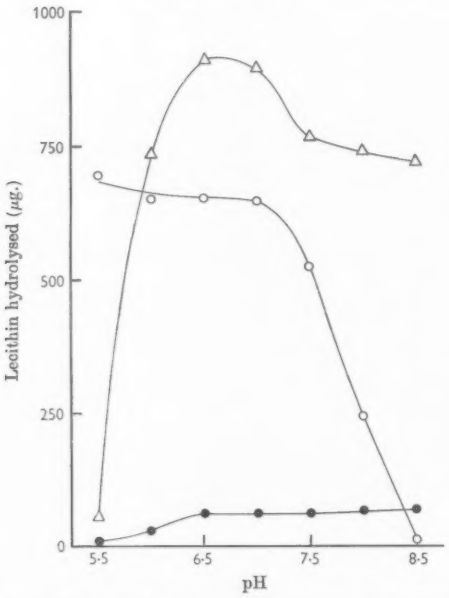

Fig. 4. Effect of pH on moccasin-venom phospholipase A. Reaction mixtures and conditions were as described in Fig. 3. ●, Effect of pH on initial reaction velocity (μg. of ovolecithin hydrolysed/min.); △, μg. of ovolecithin hydrolysed/30 min. at pH values given; ○, in these experiments, venom samples were preincubated in 0·05 M-2:4:6-collidine buffer for 4 hr. at the pH values given (temp. 30°), after which their activity was determined at pH 6·5; values shown are μg. of ovolecithin hydrolysed/15 min.

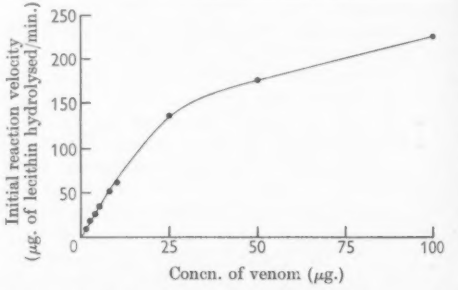

Fig. 5. Effect of venom concentration on initial reaction velocity. Reaction mixtures contained: 1 mg. of ovolecithin in 1 ml. of 2:4:6-collidine buffer (0·05 M, pH 6·5), and 0·1 ml. of ether. Venom was added in 0·05 ml. of 5 mM-$CaCl_2$. Temp. 30°.

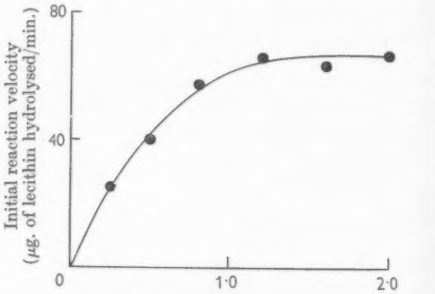

Fig. 6. Effect of substrate concentration on initial reaction velocity. Reaction mixtures contained: 1 ml. of ovolecithin emulsion (in 0·05 M-2:4:6-collidine buffer, pH 6·5), 0·1 ml. of ether and 7·5 μg. of venom added in 0·05 ml. of 5 mM-$CaCl_2$. Temp. 30°.

according to the method of Lineweaver & Burk (1934) a straight line was obtained. A Michaelis constant (K_m) of $1{\cdot}1 \times 10^{-3}$ M was calculated from the data.

Effect of Ca^{2+} *ions.* Fig. 7 shows that when $CaCl_2$ solution was not added to the reaction mixture, the rate of conversion of ovolecithin into lysolecithin was less rapid than that in a medium containing added Ca^{2+} ions. The addition of oxalate (final concentration 0·5 mM) to the $CaCl_2$-free samples lengthened the initial lag phase. The effect was more pronounced in the presence of 0·2 mM- and 0·5 mM-citrate. Ethylenediaminetetra-acetic acid (EDTA) produced a marked inhibition of the enzymic reaction at a concentration of 0·05 mM.

It was clear from the results that the system contained more Ca^{2+} ions than had been added in the $CaCl_2$ solution. Oxalate, at a concentration of 0·2 mM (not shown in Fig. 7) did not inhibit the enzymic reaction at all. The much greater inhibition produced by citrate as compared with that produced by an equivalent concentration of oxalate cannot be explained entirely on the basis of a higher calcium-binding capacity. This suggests that citrate has some other inhibitory action. It was noted above that certain buffering agents were much less satisfactory than others as reaction media. The ionic strength of the medium may play some part here, as 0·1 M-NaCl in the collidine-buffered reaction system produced an inhibition of over 50 %.

Assuming that the Ca^{2+} ion concentration in the reaction system is of the order of 0·5 mM, it is impossible to explain the marked inhibition produced by 0·05 mM-EDTA on the basis of its calcium-binding capacity alone. Possibly some other ion is required as a cofactor, or else EDTA has a direct inhibitory effect on the enzyme.

Washing the ovolecithin by the method of Folch *et al.* (1957) greatly increased the sensitivity of the assay system to added Ca^{2+} ions. Fig. 8 illustrates the dependence of initial reaction velocity (μg. of ovolecithin hydrolysed/min.) on the amount of $CaCl_2$ added to the samples. The optimum concentration of added Ca^{2+} ions in the system was 0·5 mM. Higher concentrations brought about a considerable inhibition. For example, the initial reaction velocity was decreased to 19 μg. of ovolecithin hydrolysed/min. in the presence of 150 mM-$CaCl_2$.

The calcium content (if any) of the washed ovolecithin preparation was not determined, but

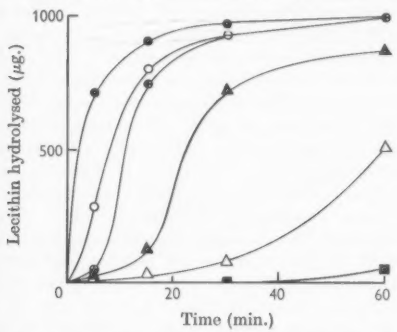

Fig. 7. Effect of Ca^{2+} ions and Ca^{2+}-complexing agents on the enzymic hydrolysis of ovolecithin by moccasin-venom phospholipase A. Reaction mixtures contained: 1 mg. of ovolecithin in 1 ml. of 2:4:6-collidine buffer (0·05 M, pH 6·5), 0·1 ml. of ether and 100 μg. of venom added in 0·05 ml. of either water or $CaCl_2$ solution. Other reagents were added individually in a volume of 0·02 ml. ●, 0·22 mM-$CaCl_2$; ○, no added $CaCl_2$; ⊕, no added $CaCl_2$, 0·5 mM-oxalate; ▲, no added $CaCl_2$, 0·2 mM-citrate; △, no added $CaCl_2$, 0·5 mM-citrate; ■, no added $CaCl_2$, 0·05 mM-EDTA. Temp. 30°.

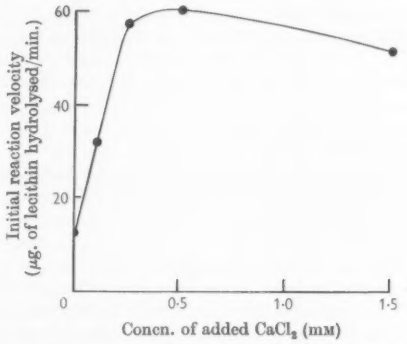

Fig. 8. Dependence of initial reaction velocity on the concentration of Ca^{2+} ions. Reaction mixtures contained: 1 mg. of ovolecithin (washed by the method of Folch, Lees & Sloane-Stanley, 1957) in 1 ml. of 2:4:6-collidine buffer (0·05 M, pH 6·5), 0·1 ml. of ether and 10 μg. of venom added in 0·05 ml. of either water or $CaCl_2$ solution. Temp. 30°.

Table 1. *Effect of certain metallic cations on the hydrolysis of ovolecithin by moccasin-venom phospholipase* A

Reaction mixtures contained: 1 mg. of washed ovolecithin in 1 ml. of 2:4:6-collidine buffer (0·05 M, pH 6·5), 0·1 ml. of ether and 10 μg. of venom. Other reagents were added in a volume of 0·02 ml. Incubation was at 30° for 30 min.

Concn. in medium of added cation (mM)	Lecithin hydrolysed (μg.)
0	47
0·5 Ca^{2+}	846
0·5 Mg^{2+}	0
0·5 Ca^{2+} + 0·5 Cu^{2+}	38
0·5 Ca^{2+} + 0·5 Zn^{2+}	173

Table 2. *Effectiveness of chloroform–methanol treatment in the extraction of ovolecithin from protein-containing mixtures*

Sample contents (in 1 ml. of collidine buffer)			Colour yield ($E_{525\,m\mu}$) Expt.		
Ovolecithin (mg.)	Pancreatin (mg.)	Assay procedure	A	B	C
0	25	Direct	0·996	1·004	1·069
0	25	Chloroform–methanol	0·031	0·033	0·034
1	0	Direct	0·462	0·477	0·452
1	0	Chloroform–methanol	0·464	0·472	0·451
1	25	Chloroform–methanol	0·439	0·460	0·438

certainly it would appear from the results to be lower than that in the untreated substrate. The concentration of calcium in the moccasin venom is not known. Under the conditions described for Fig. 8, only 15 % (150 μg.) of the ovolecithin in each sample was converted into lysolecithin after a 1 hr. incubation period when no $CaCl_2$ solution was added. The effect of added $CaCl_2$ on the washed ovolecithin preparation is also shown in Table 1.

$MgCl_2$ (0·5 mM) not only did not replace 0·5 mM-$CaCl_2$, but brought about a definite inhibition (Table 1). Confirming the results of Long & Penny (1957) both $CuCl_2$ and $ZnCl_2$ inhibited phospholipase A. The above authors reported that NaCl, KCl, $BaCl_2$, $SrCl_2$, $MgCl_2$ and $CdCl_2$ were unable to replace $CaCl_2$ in the ether reaction system.

Phospholipase A activity of commercial pancreatin. Commercial pancreatin was selected as a convenient source of phospholipase A which could be used to adapt the method to the estimation of enzyme activity in crude preparations. However, a preliminary experiment soon showed that the presence of large amounts of pancreatin in the reaction system interfered markedly with the subsequent ester linkage determination. Attempts were made with various organic solvents to extract the ovolecithin or lysolecithin or both, and not the pancreatin, from the reaction system. Finally the addition of 4 vol. of chloroform–methanol (2:1, v/v) proved satisfactory. This results in a biphasic system in which both ovolecithin and lysolecithin are quantitatively extracted into the organic phase; the pancreatin remains in the upper phase or at the boundary between the two phases. The addition of the organic solvents stops the enzymic reaction immediately.

The effectiveness of the chloroform–methanol treatment can be assessed from Table 2. In these experiments, the colour yield was determined by the ester linkage method either directly in the original samples (direct assay) or after chloroform–methanol treatment as described fully under Methods. Pancreatin alone gave a very high colour yield by the direct assay method which was decreased to a reasonable level after chloroform–methanol partitioning. Table 2 also shows that ovolecithin standards, either with or without added pancreatin, gave approximately the same colour yield (after appropriate blank and sampling corrections) after partitioning as did ovolecithin standards in which the colour was developed directly. The presence of 25 mg. of pancreatin in the samples decreased the colour yield of ovolecithin slightly, but as this effect was reasonably consistent in any one set of determinations with mixtures of lecithin and lysolecithin it was not considered to be a serious fault. With less pancreatin in the samples this effect became negligible. It was possible to use the partitioning method successfully with as much as 100 mg. of pancreatin in each sample.

Table 3. *Time course of hydrolysis of ovolecithin by pancreatin suspensions*

Reaction mixtures contained: 1 mg. of ovolecithin plus 25 mg. of pancreatin in 1 ml. of 2:4:6-collidine buffer (0·05 M, pH 6·5), 0·1 ml. of ether and 0·05 ml. of 5 mM-$CaCl_2$. Samples were incubated at 30° with constant shaking.

Reaction period (min.)	Lecithin hydrolysed (μg.) Expt. A	Expt. B
15	150	115
30	275	230
60	500	440
90	650	625
120	763	785

The time course of hydrolysis of ovolecithin by pancreatin suspensions is shown in Table 3. A plot of mean values was used to determine an initial reaction velocity of 8·9 μg. of lecithin hydrolysed/min. Comparing the phospholipase A content of moccasin venom with that of pancreatin on a specific activity basis (μg. of ovolecithin hydrolysed/hr./mg. dry wt.), it was calculated that the venom is 17 000 times as active as the pancreatin.

DISCUSSION

The method outlined, in which phospholipase A activity is determined in a reaction system containing ovolecithin in ether containing 2:4:6-collidine buffer, has been shown to be of practical use. In the presence of an active source of the enzyme, such as

the venom of the cottonmouth moccasin, the conversion of lecithin into lysolecithin proceeds rapidly and smoothly to completion under appropriate conditions. The method is simple and sensitive, and requires only small amounts of substrate. There is no lag phase in the reaction, which simplifies the determination of initial reaction velocities for kinetic purposes. This is an advantage which the buffer system has over the ether reaction system, as Long & Penny (1957) found in their experiments that there was an appreciable time lag at the start of the enzymic reaction in ether. The adjustment of pH in the aqueous buffer system is not a problem.

The degree of reproducibility of the acyl ester determination can be assessed from the fact that after the reaction is complete (e.g. after an incubation period of 1 hr. with 1 mg. of ovolecithin and 100 μg. of venom in 2:4:6-collidine buffer and other conditions as described in Fig. 1), the amount of ovolecithin hydrolysed was 1000 ± 10 μg. or 100 ± 1% (mean $\pm$ S.E.M. for eight experiments).

A comparison of the reaction velocity in the system described with that obtained by other workers is complicated by the multiplicity of different reaction conditions and assay techniques used. The slow rate of hydrolysis in ether-free buffers has already been discussed. The results of Long & Penny (1957) show that in a reaction system containing 1·5–2·0 μmoles of lecithin in 1 ml. of ether and 10 μg. of moccasin venom added in 0·01 ml. of 5 mM-calcium chloride the substrate was hydrolysed to the extent of approx. 70% after 30 min. at a temperature of 18°. With 1·2 μmoles of ovolecithin in 1 ml. of 2:4:6-collidine buffer (0·05 M, pH 6·5), 10 μg. of moccasin venom added in 0·05 ml. 5 mM-calcium chloride and 0·1 ml. of ether, 91% of the ovolecithin was converted into lysolecithin after 30 min. at a temperature of 30° (Fig. 3). Reaction rates in the two systems thus would appear to be of the same order of magnitude.

Increasing the venom concentration increased the rate of the reaction. The effect was linear with venom concentrations of from 1·25 to almost 25 μg./sample, but fell off at higher concentrations (Fig. 5). The method is applicable, therefore, to the assay of phospholipase A activity over a useful range.

With a venom concentration of 7·5 μg. and substrate concentrations ranging from 0·25 to 2·00 mg./sample a Michaelis constant of 1·1 mM was determined. Hanahan *et al.* (1954) found a K_m of 10 mM for *Naia naia* venom, but as the experimental conditions and enzyme source were different, it is not correct to compare the two values. Long & Penny (1957) noted that the venom of the cottonmouth moccasin showed a significantly higher phospholipase A activity than did *Naia naia* venom.

A requirement for Ca^{2+} ions by phospholipase A preparations from various sources has been noted previously (e.g. see Rezek, 1945; Zamecnik *et al.* 1947; Zeller, 1950; Hayaishi & Kornberg, 1954; Long & Penny, 1957; Rimon & Shapiro, 1959). The stimulation of moccasin-venom phospholipase A by Ca^{2+} ions was first studied in detail by Long & Penny (1957), who reported that the requirement for Ca^{2+} ions depended on the concentration of lecithin employed. They calculated that each μmole of ovolecithin in an ether reaction system required about 0·03 μmole of Ca^{2+} ions for maximal enzymic hydrolysis. The optimum concentration ratio of added calcium chloride in the system described in this paper was about 0·4 μmole of Ca^{2+} ions/μmole of lecithin. That more calcium chloride had to be added to the aqueous buffer system than to the ether system for maximum activity is not, perhaps, surprising, as it is unlikely that the distribution of Ca^{2+} ions in the two systems is comparable. In the ether system Long & Penny (1957) suggested that the calcium takes part in the binding of the enzyme with the substrate, and they observed that no calcium remained in the ether supernatant after the reaction was complete.

Commercial pancreatin, which on a dry-weight basis has only 0·007% of the phospholipase A activity of moccasin venom, was used to test the applicability of the method to the detection of phospholipase A activity in crude enzyme preparations. It is hoped that the method described can be used as a basis for a tissue survey of phospholipase A.

SUMMARY

1. A study has been made of the enzymic degradation of ovolecithin by snake-venom phospholipase A in a number of different buffered media.

2. A system containing 2:4:6-collidine buffer and ether is described in which the conversion of lecithin into lysolecithin proceeds rapidly to completion.

3. The course of the reaction was followed by determining the decrease in acyl ester bonds. The substrate itself was used as a standard in the method described.

4. The effect of pH, venom concentration and substrate concentration on the activity of snake-venom phospholipase A has been investigated. A Michaelis constant for the enzymic reaction was determined.

5. The optimum concentration of added Ca^{2+} ions in the reaction system was 0·5 mM.

6. The aqueous assay system has been adapted to the detection and estimation of phospholipase A activity in a crude tissue preparation (commercial pancreatin). It was calculated on a dry weight basis that the phospholipase A activity of the venom of the cottonmouth moccasin was 17 000 times as great as that of pancreatin.

We wish to thank Dr G. R. Webster and Dr R. W. R. Baker for their many helpful suggestions. We are indebted to Dr M. K. Johnson for the gift of a sample of redistilled ethanolamine. We also wish to thank the National Multiple Sclerosis Society for a Postdoctoral Fellowship to one of us (W.L.M.) and the Medical Research Council for a grant towards the expenses of this work.

REFERENCES

Chargaff, E. & Cohen, S. S. (1939). *J. biol. Chem.* **129**, 619.
Dawson, R. M. C. & Elliott, W. H. (1959). In *Data for Biochemical Research*, p. 192. Ed. by Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. Oxford: Clarendon Press.
Fairbairn, D. (1945). *J. biol. Chem.* **157**, 633.
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 143. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Habermann, E. (1957). *Biochem. Z.* **328**, 474.
Hanahan, D. J. (1952). *J. biol. Chem.* **195**, 199.
Hanahan, D. J., Rodbell, M. & Turner, L. D. (1954). *J. biol. Chem.* **206**, 431.
Hayaishi, O. & Kornberg, A. (1954). *J. biol. Chem.* **206**, 647.
Hughes, A. (1935). *Biochem. J.* **29**, 437.
Kates, M. & Gorham, P. R. (1957). *Canad. J. Biochem. Physiol.* **35**, 119.
Laidler, K. J. (1955). *Trans. Faraday Soc.* **51**, 550.
Levene, P. A. & Rolf, I. P. (1923). *J. biol. Chem.* **55**, 743.
Levene, P. A., Rolf, I. P. & Simms, H. S. (1924). *J. biol. Chem.* **58**, 859.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Long, C. & Penny, I. F. (1957). *Biochem. J.* **65**, 382.
Rapport, M. M. & Franzl, R. E. (1957). *J. biol. Chem.* **225**, 851.
Rezek, A. (1945). *Vet. Arhiv*, **15**, 161.
Rhodes, D. N. & Lea, C. H. (1957). *Biochem. J.* **65**, 526.
Rimon, A. & Shapiro, B. (1959). *Biochem. J.* **71**, 620.
Stern, I. & Shapiro, B. (1953). *Brit. J. clin. Path.* **6**, 158.
Zamecnik, P. C., Brewster, L. E. & Lipmann, F. (1947). *J. exp. Med.* **85**, 381.
Zeller, E. A. (1950). In *The Enzymes*, vol. 1, p. 988. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.

Biochem. J. (1960) **77**, 534

The Fractionation of Phosphate Esters on Ion-Exchange Resin by a new System of pH-Gradient Elution

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During a study of division lag in bacterial cultures an attempt was made to compare the acid-soluble phosphates from resting cells of *Escherichia coli* with those from rapidly dividing cells and also to compare the processes of ribonucleic acid degradation. A scheme of analysis on filter paper was devised for this purpose (Wade & Morgan, 1955) but, upon applying it to acid-soluble extracts, the constituents were found to be too numerous to fractionate and isolate in this way. Ion-exchange techniques were considered as an alternative method of achieving this.

The technique most commonly used is essentially that applied by Cohn (1950) to the fractionation of ribonucleoside 2′(3′)-phosphates. It consists of passing a solution of the phosphates through an anion-exchange resin at a pH which strongly favours adsorption and then to elute them, in order of increasing affinity for the resin, with a solution (eluent) of lower pH. It is usual to employ several eluents with progressively higher salt concentration and lower pH (Cohn & Volkin, 1953). The separation of very dissimilar phosphates is greatly simplified by changing the composition of the eluent continuously, e.g. Hurlbert, Schmitz, Brumm & Potter, 1954; Pontis & Blumson, 1958. A disadvantage of such systems is that the useful range of conditions is usually very limited and sometimes necessitates a discontinuous change in the elution programme (Hurlbert *et al.* 1954).

The system of pH-gradient elution described in this paper avoids these limitations by providing a uniform fall in eluent pH between wide limits of pH and rate of pH change. This has enabled the effects of changing the rate of pH change and the eluent salt concentration upon the fractionation of a standard mixture of ribonucleoside 2′(3′)-phosphates to be examined. The practical information gained in this way has been summarized and used to select the best conditions for the fractionations of nucleoside polyphosphates and nucleoside 2′:3′-cyclic phosphates.

MATERIALS AND METHODS

Symbols. The symbols G, S and V have been used for the rate of pH change (pH units/l. of eluent), eluent salt concentration (molarity) and resin volume (l.) respectively.

Preparation of resin column. Dowex-1 resin (Dow Chemical Co., Mich., U.S.A.) with 2% divinyl cross-linkage and 200–400 mesh was used in the chloride or formate form. It was regenerated by passing 5 vol. (resin volume) of 10% (w/v) NaCl, 2 vol. of 2% (w/v) NaOH, 2 vol. of N-HCl or N-formic acid, 2 vol. of water and 10 vol. of 1 mN-HCl or 5·3 mN-formic acid successively through it. The regenerated resin was stored in this final solution.

The resin column was prepared in the following way. A volume (10 or 20 ml.) of the resin, measured by allowing it to sediment in a measuring cylinder, was transferred to a glass column (1 cm. diam.), HCl or formic acid at pH 3 being used to wash it in. At this pH no difficulty is experienced with resin adhering to the sides of the column or failing to form a flat surface. The resin was allowed to settle in the column, the free liquid above it was drained away and a volume (1 ml.) of dry, acid-washed glass beads (no. 18, English Glass Co., Leicester) was delivered onto its surface. The resin was washed finally with 5 vol. of water (resin chloride) or 0·01 M-sodium formate (resin formate).

A much poorer resolution was obtained when the diameter of the resin column was reduced to 0·7 cm., owing to the resin settling unevenly along the length of the column and causing the eluent to channel. Another practical feature which discouraged the use of narrow columns is the high resistance of the resin to the flow of eluent.

The solutes were applied in 1 vol. (resin volume) of water (resin chloride) or 0·01 M-sodium formate (resin formate) at 0·2 ml./min.

pH *control of eluent.* Salt solution, at a progressively decreasing pH, was passed through the resin column with the system illustrated in Fig. 1.

Salt solutions passes from vessel A to vessel B (300 ml. capacity), where it is mixed, by means of the magnetic stirrer D, with acid from vessel F. The flow of acid is maintained by a Sigmamotor pump X, type T6 (Sigmamotor Inc., Middleport, N.Y., U.S.A.) and is stopped when the pH in B falls to a level determined by a control system which comprises an E.I.L. pH meter, type 23A (Electronic Instruments Ltd., Richmond, Surrey) and a Transistrol Indicating Temperature Controller, type 994B (Ether Ltd., Birmingham). The controller functions in the following way. The current from the pH meter (100 μA/pH unit) is indicated on a calibrated scale by the pointer of a galvanometer to which is attached a small vane. The vane is free to pass between a phototransistor and a small filament lamp which are both mounted on a control arm. A pointer attached to the latter indicates the required pH on the galvanometer scale, and its movement, i.e. the programme of pH change, is controlled by the shape of a celluloid cam and the speed of a synchronous motor which turns it. The motor is easily exchanged for another of different speed and a set of spur-gear wheels of different diameters allows a further adjustment of the turning speed.

A second Sigmamotor Y pumps the pH-adjusted salt solution (eluent) at 1 ml./min. from B, through the resin column, and a rotameter flow meter (0·2–2·0 ml./min. range; Rotameter Manufacturing Co. Ltd., Croydon, Surrey), which is not shown, to a time-controlled fraction collector. The speed of each pump is controlled by a Zeromax infinitely variable speed box, type 143 (Revco Inc., Minn., U.S.A.).

To ensure that data from a small-scale fractionation is sufficiently reliable to be used for the design of a large-scale preparative fractionation, an accurate synchronization of the rates of eluent flow and of pH change is essential. A constant rate of flow is not obtained with a conventional gravity-dependent system since, in the course of pH-gradient elution, the volume of the resin, and therefore its resistance to flow, changes considerably. The positive control afforded by the Sigmamotor pump is insensitive to these changes and is therefore an essential feature of the scheme.

Source of phosphates. The characteristics of pH-gradient elution were studied with the eight nucleotides produced by an alkali hydrolysis of ribonucleic acid, i.e. the ribonucleoside 2′- and 3′-phosphates of adenine, guanine, cytosine and uracil. Sodium ribonucleate from yeast (British Drug Houses Ltd., London) was hydrolysed in 0·3 N-NaOH at 37° for 18 hr. and then brought to the required pH with HCl. A small deposit which remained was filtered off and the hydrolysate was stored over chloroform at 0°.

Ribonucleoside 5′-phosphates, ribonucleoside 5′-diphosphates and ribonucleoside 5′-triphosphates were purchased from Pabst Laboratories, Milwaukee, Wis., U.S.A.

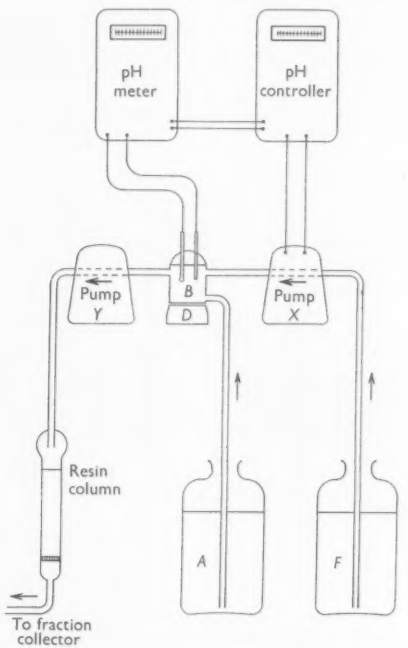

Fig. 1. The system of pH-gradient elution. A, Salt solution: B, mixing vessel; D, magnetic stirrer; F, acid solution; X and Y, Sigmamotor pumps.

Ribonucleoside 2′:3′-cyclic phosphates were kindly prepared by Mr S. Lovett by the method of Smith, Moffatt & Khorana (1958).

The quantity of material fractionated is indicated on each figure by the total quantity of phosphorus applied to the resin.

Identification of fractions in the eluate. The positions of bases and nucleotides in the eluate were determined by measuring the extinction at 260 mμ in 1 cm. cells of a Unicam spectrophotometer, type SP. 500 (Unicam Instruments Ltd., Cambridge) after suitable dilutions. The nucleotides were identified from the ratio of extinctions at 260 and 280 mμ and, when necessary, by paper ionophoresis (Wade & Morgan, 1955). The distinction between ribonucleoside 2′-phosphate and ribonucleoside 3′-phosphate was made on the assumption that the least strongly adsorbed isomer is the nucleoside 2′-phosphate (Cohn, 1950).

RESULTS AND DISCUSSION

Fractionation on 10 *ml. of resin chloride with solute displacement by hydrochloric acid starting at* pH 4. The upper useful working pH is limited to pH 4 by

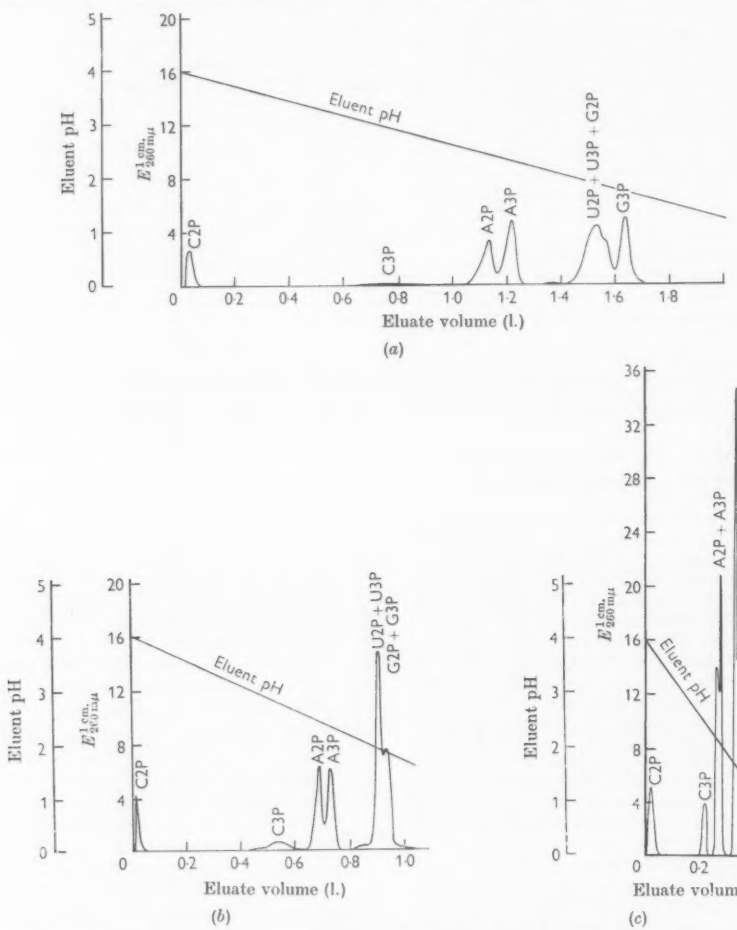

Fig. 2. Fractionation of ribonucleoside 2′(3′)-phosphates on resin chloride with hydrochloric acid solution. The nucleotides (3·5 mg. of P) were adsorbed on to 10 ml. of resin at pH 4 and eluted with hydrochloric acid solution adjusted to a pH gradient of (*a*) 1·38 pH/l., (*b*) 2·31 pH/l. or (*c*) 6·93 pH/l. C2P, Cytidine 2′-phosphate; C3P, cytidine 3′-phosphate; A2P, adenosine 2′-phosphate; A3P, adenosine 3′-phosphate; U2P, uridine 2′-phosphate; U3P, uridine 3′-phosphate; G2P, guanosine 2′-phosphate; G3P, guanosine 3′-phosphate.

the changing capacity of the resin above this pH and the difficulty of controlling the pH in an unbuffered system.

The effect of varying the rate of pH change upon the distribution and the resolution of the fractions can be seen in Fig. 2. As the rate of pH change is increased from 1·38 pH/l. of eluent to 6·95 pH/l. of eluent the relative distribution of the fractions remains unchanged and the point of elution of any one fraction is associated with a particular eluent pH. This association is more exact than is suggested by Fig. 2, for there is a time lapse of 40 min. (equivalent to 40 ml. of eluent) between the records of eluent pH and of eluate extinction. The most prominent change which occurs is an increase in the sharpness of the solute concentration peaks, but this is not accompanied, however, by an improvement in the resolution. The behaviour of the two adenine isomers illustrates this point (Fig. 2).

Fractionation on 10 ml. of resin chloride with solute displacement by sodium chloride–hydrochloric acid solution starting at pH 4. The effects of including sodium chloride in the eluent at the pH gradient used in Fig. 2 (*b*) can be seen in Figs. 3 (*a*), 3 (*b*) and 3 (*c*). Increasing the salt concentration from

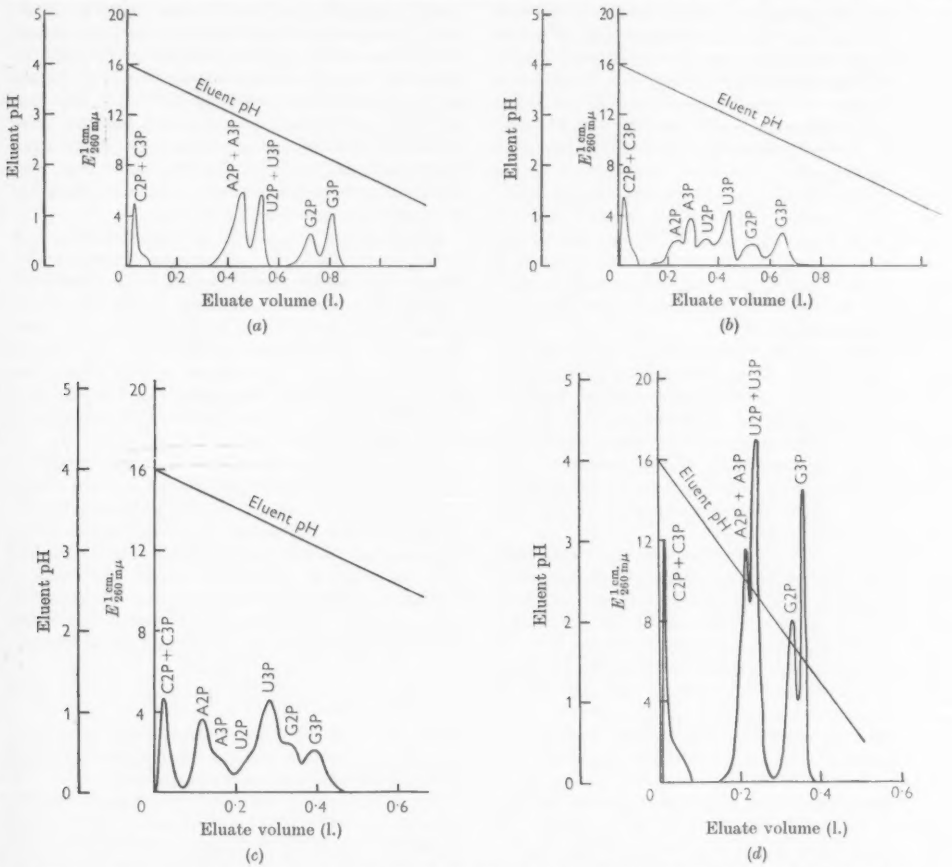

Fig. 3. Fractionation of ribonucleoside 2′(3′)-phosphates on resin chloride with sodium chloride solution adjusted with hydrochloric acid solution. The nucleotides (3·5 mg. of P) were adsorbed on to 10 ml. of resin at pH 4 and eluted with a pH gradient either of 2·31 pH/l. with (*a*) 0·01 M-sodium chloride, (*b*) 0·02 M-sodium chloride or (*c*) 0·05M-sodium chloride solution, or of (*d*) 6·95 pH/l., with 0·02 M-sodium chloride solution. C2P, Cytidine 2′-phosphate; C3P, cytidine 3′-phosphate; A2P, adenosine 2′-phosphate; A3P, adenosine 3′-phosphate; U2P, uridine 2′-phosphate; U3P, uridine 3′-phosphate; G2P, guanosine 2′-phosphate; G3P, guanosine 3′-phosphate.

0·01 to 0·05 M alters the relative distributions of the fractions but has very little influence upon the sharpness of the peaks. A comparison between Fig. 2 (*c*) and Fig. 3 (*c*), in both of which the fractionation is complete with 500 ml. of eluate, illustrates the better resolution obtained by increasing the rate of pH change rather than the eluent salt concentration.

When 0·02 M-sodium chloride is used and the pH gradient is increased from 2·31 (Fig. 3*b*) to 6·95 pH/l. (Fig. 3*d*), the sharpness of the peaks is increased, as expected, but, unlike a similar change in the absence of salt from the eluent (Figs. 2*b* and 2*c*), the distribution is altered. The resulting distribution (Fig. 3*d*) resembles neither that produced by a similar pH gradient in the absence of salt (Fig. 2*c*) nor that produced by a similar salt concentration with a different pH gradient (Fig. 3*b*). It resembles more closely the distribution produced by 0·01 M-sodium chloride with a pH gradient of 2·31 pH/l. (Fig. 3*a*). The only other feature which distinguishes these fractionations (Figs. 3*a* and 3*d*) from the others is the ratio of the pH gradient to the eluent salt concentration. Although these ratios are not identical in Figs. 3 (*a*) and 3 (*d*), they are of the same order of magnitude. The interdependence of pH gradient and eluent salt concentration suggested by these results is examined more fully later with a buffered system.

Difficulties of pH *control with a resin chloride system above* pH 4. A number of mixtures such as the nucleoside di- or tri-phosphates can be fractionated successfully by pH-gradient elution on a resin chloride with an unbuffered eluent starting at pH 4. For the separation of less strongly acidic phosphates, however, a higher starting pH is necessary and certain difficulties, less serious at lower pH values, become more acute.

The adsorption of phosphates on to a resin chloride in solutions of weak buffering capacity below pH 4 results in wide variations in eluate pH which are reflected in the behaviour of the less acidic solutes. When solutes are applied to the resin at pH 4 and the elution is started at this pH, the eluate pH falls rapidly to about pH 2·8 and then slowly rises to about the same level as the eluent pH. The probable explanation is that chloride ions on the resin are displaced by less than an equivalent of phosphate ions. When nucleoside 2′(3′)-phosphates are fractionated, the practical outcome is that cytidine 2′-phosphate is not adsorbed on to the resin. A similar result is obtained when the mixture is applied at pH 5 or pH 6. At pH 8 there is sufficient increase in resin capacity between pH 8 and pH 4 to avoid a pH fall below pH 4, with the result that the cytidine 2′-phosphate becomes adsorbed and is later eluted as a distinct solute peak like its isomer.

Another practical difficulty, when the elution is started above pH 4, is that the eluate pH falls initially at a much slower rate than the eluent pH and then falls rapidly when the increasing capacity of the resin is finally overcome. This results in the close grouping of the solutes in the eluate during the latter period. A similar situation arises in a buffered system when the rate of pH change, relative to the eluent salt concentration, is too high (Fig. 6*b*).

Ideally, the difference in pH between the eluent and eluate should remain nearly constant. When a resin chloride is used an improvement to this end is obtained by using one or more buffers in the eluent. The separation of orthophosphate, pyrophosphate, adenosine 5′-monophosphate, adenosine 5′-diphosphate and adenosine 5′-triphosphate, for example, can be achieved by including formate in the eluent. An improvement is not invariably obtained by including a buffer in the eluent, however, for the latter sometimes becomes adsorbed on to the resin at a high pH and later resists a pH fall at a lower value. A better control of pH is obtained by using a weak acid on the resin and in the eluent. This enables a uniform fall in eluate pH to be obtained at relatively low salt concentrations.

Fractionation on 20 ml. of resin formate with solute displacement by ammonium formate–hydrochloric acid solution starting at pH 5. The better pH control afforded by a buffered eluent allows the solutes to be delivered on to the resin at pH 6 and the elution to be started at pH 5 or 6. In order to avoid unnecessary delay in the elution of the first fraction which would otherwise occur, the elution of the standard mixture of nucleoside 2′(3′)-phosphates was started at pH 5.

It has already been observed in an unbuffered system that the rate of pH change and the eluent salt concentration are somehow interdependent with respect to their influences upon the relative distribution of the fractions in the eluate. The results of a large number of fractions carried out with a formate-buffered system showed that the distribution is largely determined by the ratio (R) of the rate of pH change to the eluent salt concentration:

$$R = \frac{GV}{S}, \qquad (1)$$

where G is the rate of pH change (pH units/l. of eluent), V is the wet volume of the resin (l.) and S is the eluent salt concentration (molarity).

Fig. 4 illustrates the relative constancy of the distribution when R is kept constant. The marked influence of R upon the point of elution of individual fractions and upon the distribution is illustrated in Fig. 5.

The reason for the sudden deterioration in the resolution above R 1·6 (Fig. 5) can be seen in Fig. 6, in which the results of fractionating at R 1·38 and R 2·76 are compared in detail. At the higher value

(Fig. 6*b*) the eluent is unable to accommodate the increasing capacity of the resin and the association of the liberated formic acid, without itself undergoing a marked change in pH. The sudden drop in pH of the eluate when the changing capacity of the resin is finally overcome, causes the close grouping of the fractions and a very poor resolution of the mixture.

The interdependence of G and S with respect to their effects upon the eluate pH and solute distribution can be explained in the following way. In the period taken for a given fall in eluent pH, a certain equivalent of anion is required by the resin to satisfy the increase in its capacity created by the increased dissociation of its basic groups and the association of the formate ions attached to it. If the eluent is unable to supply this equivalent without suffering a serious change in pH then one of two remedies is available. The anion concentration supplied can be increased to a satisfactory level either by increasing the quantity of anion in the volume of eluent passing through the resin during this period, i.e. by increasing S, or by retaining the existing value of S and increasing the volume, i.e. by decreasing G. The same end is achieved by increasing S or decreasing G by a common factor (equation 1).

The small changes in the distribution of solutes which are observed when S is varied and R is kept constant (Fig. 4) are probably due to changes in the capacity of the resin brought about by changes in the ionic strength of the eluent. If this supposition is correct then increasing the eluent salt concentration from 0·02 M to 0·08 M should be equivalent to increasing V or R (equation 1). Conversely, decreasing the concentration from 0·02 M to 0·01 M should be equivalent to decreasing R. A comparison of Figs. 4 and 5 shows that this does in fact happen with the adenosine and guanosine phosphate; increasing the salt concentration in Fig. 4 shows to a smaller degree the same effect as increasing R in Fig. 5. The absence of any similar effect with cytidine and uridine phosphates can be attributed to the over-riding influence of the starting pH, which has been found to influence the elution of the less-strongly adsorbed solutes in a mixture.

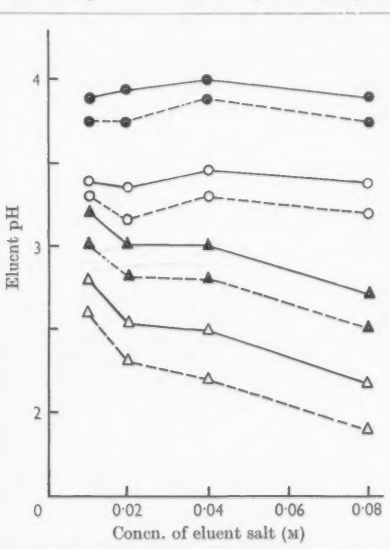

Fig. 4. Effect of varying the eluent salt concentration at R 1·38 upon the relative distribution of the fractions in the eluate. Ribonucleoside 2′(3′)-phosphates (1·75 mg. of P) were adsorbed on to 20 ml. of resin formate at pH 6 and eluted with solutions containing different concentrations of ammonium formate adjusted with hydrochloric acid. The rate of pH change was adjusted, at each level of salt concentration, to maintain R at 1·38. ●—●, Cytidine 2′-phosphate; ●- - -●, cytidine 3′-phosphate; ○—○, uridine 2′-phosphate; ○- - -○, uridine 3′-phosphate; ▲—▲, adenosine 2′-phosphate; ▲- - -▲, adenosine 3′-phosphate; △—△, guanosine 2′-phosphate; △- - -△, guanosine 3′-phosphate.

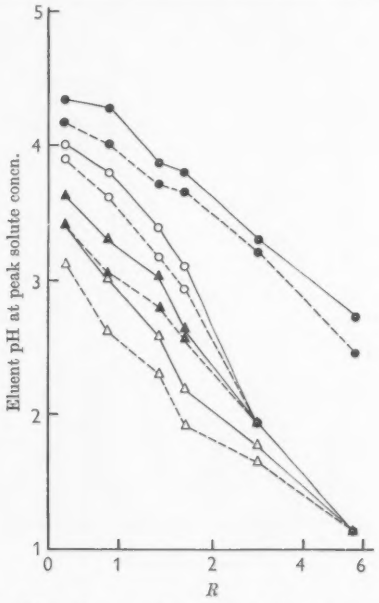

Fig. 5. Effect of varying R upon the relative distribution of the fractions. Ribonucleoside 2′(3′)-phosphates (1·75 mg. of P) were adsorbed on to 20 ml. of resin formate at pH 6 and eluted with 0·02 M-ammonium formate adjusted with hydrochloric acid to different rates of pH change. ●—●, Cytidine 2′-phosphate; ●- - -●, cytidine 3′-phosphate; ○—○, uridine 2′-phosphate; ○- - -○, uridine 3′-phosphate; ▲—▲, adenosine 2′-phosphate; ▲- - -▲, adenosine 3′-phosphate; △—△, guanosine 2′-phosphate; △- - -△, guanosine 3′-phosphate.

Fractionation of a mixture of ribonucleoside 5′-mono, di- and tri-phosphates. With the information gained from the fractionation of nucleoside 2′(3′)-phosphates, an attempt was made to separate a mixture of ribonucleoside 5′-monophosphates and ribonucleoside 5′-polyphosphates. The upper useful limit of R (R 1·6) imposed by changes in the anion capacity of the resin was further restricted by the acid-lability of the nucleoside polyphosphates. At high values of R there is a danger that these phosphates will be hydrolysed by the low pH values required to elute them.

The influence of R upon the relative distribution of fractions in the eluate was first examined at the levels 0·138, 0·276, 0·55 and 1·38. A plot of the distributions obtained (Fig. 7) suggested that a value of R 0·43 would provide an adequate resolution of the mixture and this was confirmed when the fractionation was carried out (Fig. 8).

The separation of some of these nucleotides has been described by Hurlbert *et al.* (1954) in a discontinuous form of gradient elution and also by Pontis & Blumson (1958).

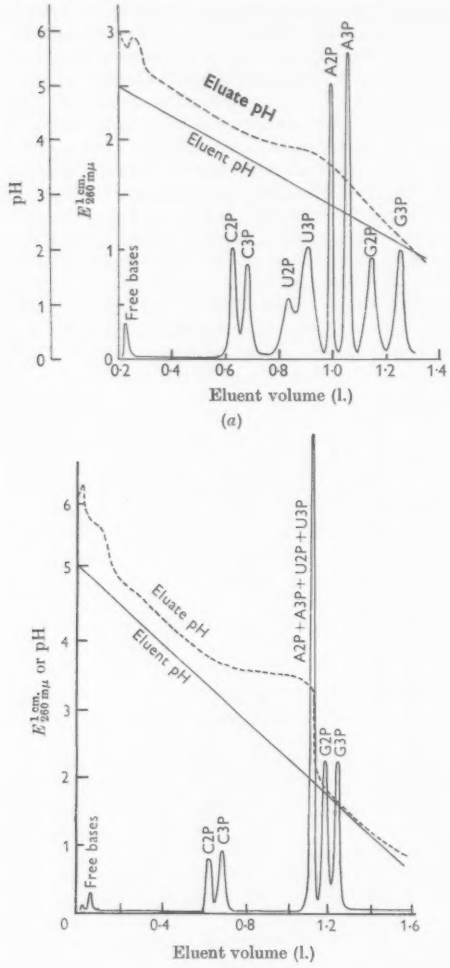

Fig. 6. Deterioration in resolution as R increases. Ribonucleoside 2′(3′)-phosphates (1·75 mg. of P) were adsorbed on to 20 ml. of resin formate at pH 6 and eluted with 0·02 M-ammonium formate adjusted with hydrochloric acid to a pH gradient of (*a*) 1·38 pH/l. (R 1·38) and (*b*) 2·76 pH/l. (R 2·76). C2P, Cytidine 2′-phosphate; C3P, cytidine 3′-phosphate; A2P, adenosine 2′-phosphate; A3P, adenosine 3′-phosphate; U2P, uridine 2′-phosphate; U3P, uridine 3′-phosphate; G2P, guanosine 2′-phosphate; G3P, guanosine 3′-phosphate.

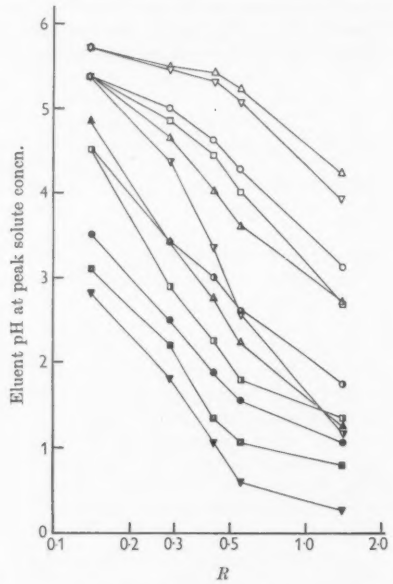

Fig. 7. Effect of varying R upon the relative distribution of ribonucleoside 5′-phosphates. The nucleotides (about 0·8 mg. of P) were adsorbed on to 20 ml. of resin formate and eluted with ammonium formate solution adjusted with hydrochloric acid to a pH gradient of 2·76 pH/l. The value of R was varied by varying the concentration of the eluent salt solution. △, Cytidine 5′-phosphate; ▲, cytidine 5′-diphosphate; ▲, cytidine 5′-triphosphate; ○, adenosine 5′-phosphate; ◑, adenosine 5′-diphosphate; ●, adenosine 5′-triphosphate; ▽, uridine 5′-phosphate; ▼, uridine 5′-diphosphate; ▼, uridine 5′-triphosphate; □, guanosine 5′-phosphate; ◧, guanosine 5′-diphosphate; ■, guanosine 5′-triphosphate.

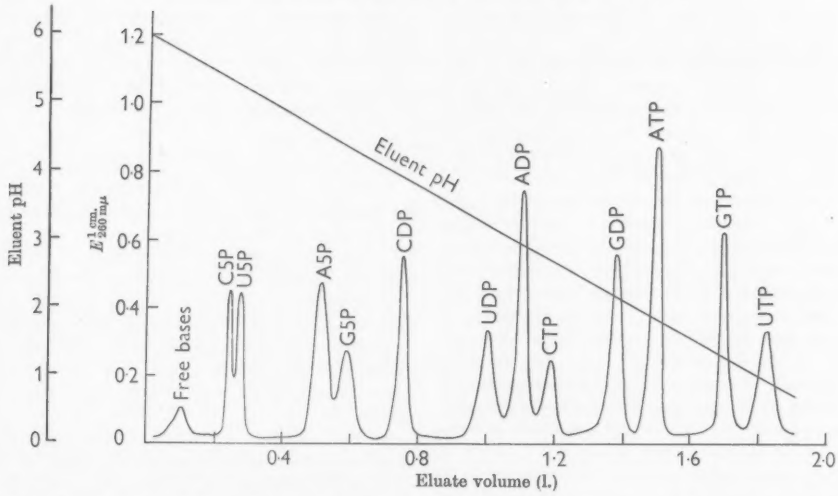

Fig. 8. Fractionation of ribonucleoside 5′-phosphates on resin formate. Ribonucleoside 5′-monophosphates (0·1 mg. of P), ribonucleoside 5′-diphosphates (0·5 mg. of P) and ribonucleoside 5′-triphosphates (1 mg. of P) were adsorbed on to 20 ml. of resin and eluted with 0·13 M-ammonium formate solution adjusted with hydrochloric acid to a pH gradient of 2·76 pH/l. (R 0·43). C5P, Cytidine 5′-phosphate; CDP, cytidine 5′-diphosphate; CTP, cytidine 5′-triphosphate; A5P, adenosine 5′-phosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; U5P, uridine 5′-phosphate; UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate; G5P, guanosine 5′-phosphate; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate.

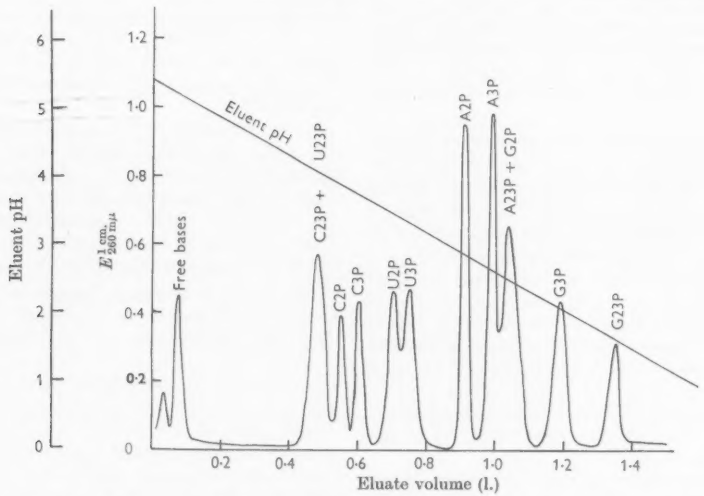

Fig. 9. Fractionation of ribonucleoside 2′:3′-cyclic phosphates and ribonucleoside 2′(3′)-phosphates on resin formate. The nucleotides (1 mg. of P) were adsorbed on to 20 ml. of resin and eluted with 0·04 M-ammonium formate solution adjusted with hydrochloric acid to a pH gradient of 2·76 pH/l. (R 1·38). C2P, Cytidine 2′-phosphate; C3P, cytidine 3′-phosphate; C23P, cytidine 2′:3′-cyclic phosphate; U2P, uridine 2′-phosphate; U3P, uridine 3′-phosphate; U23P, uridine 2′:3′-cyclic phosphate; A2P, adenosine 2′-phosphate; A3P, adenosine 3′-phosphate; A23P, adenosine 2′:3′-cyclic phosphate; G2P, guanosine 2′-phosphate; G3P, guanosine 3′-phosphate; G23P, guanosine 2′:3′-cyclic phosphate.

Fractionation of a mixture of nucleoside 2′(3′)-phosphates and nucleoside 2′:3′-cyclic phosphates. The results illustrated in Fig. 5 suggest that when the elution programme is started at pH 5 the most satisfactory value of R for the separation of nucleoside 2′-phosphates and nucleoside 3′-phosphates is about 1·38 (Fig. 6*a*). The nucleoside 2′:3′-cyclic phosphates can also be separated from one another under these conditions. The separation of an individual cyclic nucleotide from the corresponding nucleoside dihydrogen phosphates, however, cannot be obtained in this way for there is a coincidence between cytidine 2′-phosphate and cytidine 2′:3′-cyclic phosphate and also between cytidine 3′-phosphate and uridine 2′:3′-cyclic phosphate. It had been observed previously that the relative distribution of the less strongly absorbed fractions is influenced by the starting pH of the elution. By starting the fractionation at pH 5·5 these separations could be achieved (Fig. 9).

The separation of nucleoside 2′(3′)-phosphates in an alkali hydrolysate of RNA with a system of four eluents has been described by Cohn & Volkin (1951). The separation of purine 2′(3′)-phosphates by a gradient system has been described by Hurlbert *et al.* (1954). The separation of cytidine, uridine and guanosine 2′:3′-cyclic phosphates from their respective 2′-phosphates and 3′-phosphates with single eluent systems has been described by Brown, Dekker & Todd (1952).

Features of practical significance in the pH-*gradient elution of phosphates.* The following generalizations have been drawn from the results of a large number of fractionations and are presented as a practical guide.

Unless the fractionation is to be carried out below pH 3, a weak acid form of the resin should be used in conjunction with an eluent containing the salt of the same acid.

Above pH 3, where

$$R = \frac{GV}{S}$$

and G is the rate of pH change, V is the resin volume and S is the eluent salt concentration, the upper useful limit of R should be determined and not exceeded.

The variation of R below this limit affords a means of varying the relative distribution of the fractions in the eluate.

Within certain limits, the time taken to carry out a fractionation can be varied, without seriously altering the relative distribution of the fractions, by changing G and S while keeping R constant and retaining the same starting pH.

At any value of R the distribution of the less strongly adsorbed solutes can be altered by changing the starting pH.

The sharpness of a concentration peak in the eluate is increased by increasing G and is not influenced by S.

At a given value of R the degree of resolution of a mixture increases as G and S decrease.

SUMMARY

1. A system of pH-gradient elution is described for the fractionation of naturally occurring phosphate esters on ion-exchange resin.

2. The method uses a direct form of pH control and offers a wide choice in conditions of elution.

3. The effects of varying the rate of pH change and the eluent salt concentration upon the relative distribution of the fractions and upon the sharpness of their concentration peaks have been examined with a standard mixture of nucleoside 2′(3′)-phosphates.

4. The relative distribution of the solutes in the eluate is largely determined by the ratio (R) of the rate of pH change to eluent salt concentration [$R = (GV)/S$, where G is the rate of pH change in pH units/l. of eluent, V is the wet volume of resin and S is the eluent salt concentration]. The time taken for a fractionation can be varied, without radically changing the distribution, by varying these values without changing R.

5. The sharpness of the solute concentration peak is determined by the rate of pH change and is not influenced by the eluent salt concentration.

6. On a standard column the resolution of a mixture at any given level of R is improved by decreasing the rate of pH change and the eluent salt concentration.

7. In addition to the fractionation of ribonucleoside 2′(3′)-phosphates, the fractionations of ribonucleoside 5′-mono-, di- and tri-phosphates and of ribonucleoside 2′:3′-cyclic phosphates are described.

8. From the results of a large number of fractionations, several generalizations have been made and are presented as a practical guide.

I am pleased to record my thanks to Mr D. M. Morgan and Mr C. D. Kimber for their technical assistance and to Dr D. Herbert for advice.

REFERENCES

Brown, D. M., Dekker, C. A. & Todd, A. R. (1952). *J. chem. Soc.* p. 2715.

Cohn, W. E. (1950). *J. Amer. chem. Soc.* **72**, 1471.

Cohn, W. E. & Volkin, E. (1951). *Nature, Lond.*, **167**, 483.

Cohn, W. E. & Volkin, E. (1953). *J. biol. Chem.* **203**, 767.

Hurlbert, R. B., Schmitz, H., Brumm, A. F. & Potter, Van R. (1954). *J. biol. Chem.* **209**, 23.

Pontis, H. G. & Blumson, N. L. (1958). *Biochim. biophys. Acta*, **27**, 618.

Smith, M., Moffatt, J. G. & Khorana, H. G. (1958). *J. Amer. chem. Soc.* **80**, 6204.

Wade, H. E. & Morgan, D. M. (1955). *Biochem. J.* **60**, 264.

Biochem. J. (1960) **77**, 543

β-Pyrazol-1-ylalanine, an Amino Acid from Water-Melon Seeds (*Citrullus vulgaris*)

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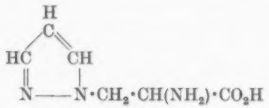
Paper chromatography has been used extensively to study the free amino acids present in a wide variety of plant species. Comprehensive reviews describe the chemical and biological properties of many of these acids (Virtanen, 1957; Fowden, 1958, 1960; Hunt, 1959). This paper describes the isolation and identification of another acid, β-pyrazol-1-ylalanine (I), which forms a major component of the soluble nitrogen fraction of water-melon seeds. A preliminary account has been given by Noe & Fowden (1959). The possibility that pyrazole is used directly in the synthesis of the amino acid has been investigated by supplying young melon seedlings with pyrazole alone and in association with various amino acids.



(I)

ISOLATION AND IDENTIFICATION

The substance was encountered first as an unidentified ninhydrin-reacting spot on paper chromatograms prepared from 75 % (v/v) ethanol extracts of ground water-melon seeds (*Citrullus vulgaris* var. Tom Watson). About 3 g. of the substance was isolated from an extract of 4·5 kg. of seeds. The empirical formula, $C_6H_9O_2N_3$, suggested that the substance was an isomer of histidine. The presence of an α-amino group was indicated by the compound's ability to chelate copper. On paper chromatograms it gave a yellow colour with Ehrlich reagent, and reacted slowly to give a blue colour after treatment with isatin. The Pauly reaction was negative. Degradation with hydriodic acid yielded alanine and what was probably an iodo derivative of a heterocyclic base which was not imidazole. The isolated material had a pK_A value of 2·2. This favoured the idea of a pyrazole nucleus since the pK_A values associated with the protonation of the tertiary nitrogen atom of imidazole and pyrazole are 7·03 and 2·53 respectively (Jacobs, 1957).

Nuclear magnetic resonance spectroscopy (see Appendix) established that a pyrazole ring formed the heterocyclic component of the amino acid and that the alanine group was attached to the ring at a nitrogen and not at a carbon atom. The production of alanine during treatment with hydriodic acid provided other evidence in favour of the nitrogen–carbon linkage; this type of bond would be expected to undergo reductive fission far more readily than a carbon–carbon bond. Confirmation of the structure was obtained by synthesis of β-pyrazol-1-ylalanine, which was identical with the racemized natural product. The compound was readily separated from synthetic β-imidazol-1-ylalanine on paper chromatograms developed with butanol–acetic acid–water.

Shinano & Kaya (1957) isolated from press-juice of water melon a small quantity of an amino acid having the same empirical formula and m.p. as β-pyrazol-1-ylalanine. Noting that their compound was isomeric with histidine, they suggested that the amino acid was β-imidazol-1-ylalanine. Since water melon was the plant tissue used by Shinano & Kaya and in the present investigation, the two substances isolated were almost certainly identical, and, in view of the more critical evidence now presented, the assignment of the imidazolylalanine structure must be regarded as incorrect.

β-Pyrazol-1-ylalanine forms one of a group of aromatic or heterocyclic amino acids derived from alanine that have been characterized recently as higher-plant components. The group includes 2:4-dihydroxy-6-methylphenylalanine, isolated from seeds of *Agrostemma githago* (Schneider, 1958), β-(3-carboxy-γ-pyron-3-yl)alanine (stizolobic acid) isolated from *Stizolobium hassjoo* seedlings (Hattori & Komamine, 1959) and β-(uracil-3)-alanine (willardiine), isolated from seed of *Albizzia willardia* (Gmelin, 1959). In the last-named substance, the alanine is attached to a nitrogen atom of the uracil ring.

DISTRIBUTION

β-Pyrazol-1-ylalanine was the major free amino acid (approx. 1 g./kg.) in dry water-melon seeds. Citrulline (approx. 0·15 g./kg.) and glutamic acid (approx. 0·1 g./kg.) were also readily detected by chromatographic methods. During the early phase of seedling growth in the dark, many other free amino acids appeared as products of protein degra-

dation. Although some increase in the total amount of the pyrazolylalanine present in a seedling occurred during growth, concentrations of several other amino acids soon exceeded that of the pyrazolylalanine. β-Pyrazol-1-ylalanine is particularly characteristic of the dormant seed, and forms only a trace component of the vegetative tissues.

β-Pyrazol-1-ylalanine was detected as a minor component of extracts prepared from several other seeds of the family Cucurbitaceae, e.g. *Cucurbita pepo* var., *Bligia sapida* Kon. (vegetable marrow), *Cucurbita ficifolia*, *Cucumis melo* (melon), *Cucumis sativa* (cucumber), *Ecballium elaterium* (squirting cucumber), *Bryonia dioica* (white bryony) and *Echinocystis lobata*. The acid was present in these species in concentrations considerably less than that found in water-melon seed.

Data on the free amino acid fraction of numerous plant species indicate that β-pyrazol-1-ylalanine is not generally distributed in plants. Like many other recently identified non-protein amino acids, it may be confined to a relatively few plant species, of which the only examples so far known belong to the family Cucurbitaceae. However, the study of the free amino acid composition of plants has been directed largely to vegetative tissues and since the pyrazolylalanine has been found predominantly in seeds, studies on a wider range of seeds may reveal that this amino acid occurs in other families of plants.

BIOSYNTHESIS

The identification of β-pyrazol-1-ylalanine as a component of certain plants has added to the main types of naturally occurring heterocyclic compounds. There appears to be no previous report of the pyrazole-ring system occurring in natural products. Although the biosynthetic pathway leading to the formation of the pyrazole ring is unknown, biosynthesis from compounds such as hyponitrite or hydrazine, which contain linked nitrogen atoms, seems possible since several chemical syntheses of pyrazole utilize hydrazine as a starting material (Jacobs, 1957).

To test the possibility that pyrazole may be used directly for synthesis of β-pyrazol-1-ylalanine, seeds of *Cucumis melo* were used because they germinated more rapidly and consistently than the water-melon seed available. The seeds, after surface sterilization with weak sodium hypochlorite solution, were allowed to germinate in Petri dishes at 30° in the presence of the sterile solutions listed in Table 1. After 2 days, when the radicle had grown to about 1 cm. length, the seeds were planted in moistened vermiculite and grown for a further 5 days in the dark at 30°. The seeds were then killed and extracted with 75% (v/v) ethanol and the amounts of various amino acids present in each extract were determined by the paper-chromatographic technique of Fowden & Webb (1958) and Bryant & Fowden (1959). The results in Table 1 show that pyrazole may be incorporated, presumably as the intact ring system, into β-pyrazol-1-ylalanine. The quantity of the amino acid in seedlings supplied with pyrazole was approximately double that of similar plants not receiving pyrazole (Table 1). There was no further increase when alanine or serine was supplied in association with pyrazole. The results therefore do not support the idea that either alanine or serine is incorporated directly into the side chain of β-pyrazol-1-ylalanine in a manner similar to that by which tryptophan is known to be synthesized from indole and serine in higher plants (Polyanovskii & Kretovich, 1957; Mudd & Zalik, 1958; Greenberg & Galston, 1959). However, a more crucial test of this hypothesis is required.

EXPERIMENTAL

Chromatographic techniques. Paper chromatograms were run on Whatman no. 3 MM chromatographic-grade filter paper. The solvents used included: A, 75% (w/v) phenol solution in the presence of NH_3 solution; B, a one-phase mixture of butan-1-ol–acetic acid–water (90:10:29, by vol.); C, 70% (v/v) propan-1-ol; D, the upper phase of ethyl acetate–pyridine–water (2:1:2, by vol.); E, butan-1-ol saturated with 2N-NH_3 soln. and F, propan-1-ol–0·2N-NH_3 solution (3:1, v/v). A 0·1% (w/v) solution of ninhydrin in 95% (v/v) ethanol was normally used as a spraying agent.

Table 1. *Concentrations of β-pyrazol-1-ylalanine in* Cucumis *seedlings supplied with various nitrogenous compounds during the first 2 days' growth*

Results are expressed as μg. of α-amino N present in pyrazolylalanine/g. fresh wt. of tissue.

Treatment	Concn. of pyrazolylalanine	Increase in concn. above that of appropriate control
Distilled water	8·1	—
0·125% Pyrazole	16·9	8·8
0·125% Pyrazole + 0·25% DL-alanine	15·2	8·8
0·125% Pyrazole + 0·25% DL-serine	15·4	9·1
0·25% DL-Alanine	6·4	—
0·25% DL-Serine	6·3	—

Occasionally the isatin, Sakaguchi, Ehrlich and persulphate dipping reagents of Jepson & Smith (1953*a*, *b*), were used.

Detection of β-pyrazol-1-ylalanine in water-melon seed. Paper chromatograms of aq. 75% (v/v) ethanol extracts of ground-seed material showed the presence of the pyrazolylalanine, giving a normal bluish purple colour with ninhydrin, that moved to a position nearly coincident with that of proline when solvent A followed by solvent B were used for development. The pyrazolylalanine was readily separated from the other amino acids of the seed extract on one-dimensional chromatograms in solvent B, and so its reactivity with reagents other than ninhydrin was easily checked. The pyrazolylalanine gave a strong yellow spot with Ehrlich reagent and reacted insensitively with isatin to produce a blue spot. No colour was produced on treatment with the Sakaguchi, persulphate or Pauly reagents. The amino acid chelated copper from a solution of copper acetylacetonate in chloroform.

Isolation of β-pyrazol-1-ylalanine. Ground water-melon seed (4·5 kg.) was stirred with 70 l. of aq. 75% (v/v) ethanol at 40–60° for 6 hr. and for a further 60 hr. at laboratory temperature. After filtration through Celite, the extract was evaporated *in vacuo* at about 35° to 4·5 l. Lipid material was extracted by shaking with 2 l. of ether, and the aqueous phase was adjusted to about pH 4·0 and diluted to 10 ml. The diluted extract was applied at a rate of 500 ml./hr. to a Zeo-Karb 215 (resin mesh 80–100) column, 80 cm. long, 6 cm. diam.

All the amino acids were absorbed. The column was washed with 20 l. of distilled water and eluted with 0·25 N-NH_3 solution (12 l.). When amino acids appeared in the eluate, 20 ml. fractions were collected. Resolution was not good, the pyrazolylalanine being present in a large proportion of the fractions, together with smaller amounts of other amino acids. These fractions were pooled and evaporated *in vacuo* to about 500 ml. The concentrate was treated with activated charcoal (Partridge, 1949) and then evaporated to about 100 ml. On cooling, the crude amino acid separated out as a white solid. After recrystallization from water, about 2 g. of pure β-pyrazol-1-ylalanine was obtained. A further quantity was obtained by treating the combined mother liquors on a column of Dowex-50 × 4 (mesh 100–200), 40 cm., diam. 2 cm. The amino acid was in the earliest ninhydrin-positive fractions eluted from this column. These fractions, which contained only traces of other amino acids, were combined and evaporated *in vacuo* to 30 ml. The crude material which separated on cooling was recrystallized from aq. 50% (v/v) ethanol to yield the pure amino acid (about 1 g.).

Properties. β-Pyrazol-1-ylalanine had m.p. 236–238° and $[\alpha]_D^{20}$ −73° in water (*c*, 3·4) (Found: C, 46·7; H, 5·8; N, 27·1. $C_6H_9O_2N_3$ requires C, 46·4; H, 5·8; N, 27·1%). When an 8 mM solution was titrated against 0·1 N-HCl, a pK_A of 2·2 was obtained, corresponding to the protonation of the tertiary nitrogen atom of the pyrazole ring. Histidine, titrated under comparable conditions, had an equivalent pK_A of 6·1. On paper electrophoresis the pyrazolylalanine at pH 7·0 moved towards the anode, whereas histidine remained at the origin.

Degradation. β-Pyrazol-1-ylalanine was completely destroyed when a 1% (w/v) solution of the amino acid in 55% (w/w) HI was heated at 120–130° for 18 hr. After removal of HI by repeated evaporation to dryness *in vacuo*, portions of the reaction mixture were applied to paper chromatograms developed in all six solvents. Two spots were revealed on all chromatograms after treatment with ninhydrin. The slower-moving, bluish purple spot was inseparable from added alanine in all six solvents. The second spot, which was yellow–brown in colour, travelled at similar rates to the product obtained when pyrazole was treated with 55% (w/w) HI under comparable conditions. The second spot was probably given by an iodopyrazole (many reagents readily bring about substitution on the C-4 atom of the pyrazole ring).

Synthesis of β-pyrazol-1-ylalanine. The methyl ester of β-chloroalanine hydrochloride (25 g.), prepared by the method of Fischer & Raske (1907), was refluxed with 62 g. of silver pyrazole in 400 ml. of methanol for 48 hr. After removal of methanol, 500 ml. of 6 N-HCl was added and the mixture was refluxed for 2 hr. Precipitated AgCl was removed by filtration and thoroughly washed with hot water. The filtrate and washings were combined and evaporated to a sticky residue containing the pyrazolylalanine, serine and a little alanine. The pyrazolylalanine was separated from the other two amino acids on a Dowex-50 column; yield was about 0·2 g. (approx. 1% of theoretical), m.p. 236–238° (decomp.) (Found: C, 46·5; H, 5·9; N, 27·8. $C_6H_9O_2N_3$ requires C, 46·4; H, 5·8; N, 27·1%). For a better synthetic method see Finar & Utting (1960).

Identity of the natural and synthetic amino acids. The two substances showed identical chromatographic behaviours in all six solvents listed above. The nuclear magnetic resonance spectra of the two substances dissolved in strong sodium deuteroxide solution showed the same absorption maxima. The natural acid (500 mg.) was racemized by refluxing with 5 ml. of 5 N-$Ba(OH)_2$ solution for 36 hr. After removal of barium as the sulphate and carbonate, 150 mg. of DL-acid (a 2% solution in water showed no optical rotation) was obtained after crystallization. This racemized natural acid had an infrared spectrum identical with that of the synthetic acid (Fig. 1). The differences between the infrared spectra of the natural (−)-acid and those of either the racemized or synthetic preparations were quite small, and consisted mainly of small shifts in the frequencies of the major absorption peaks.

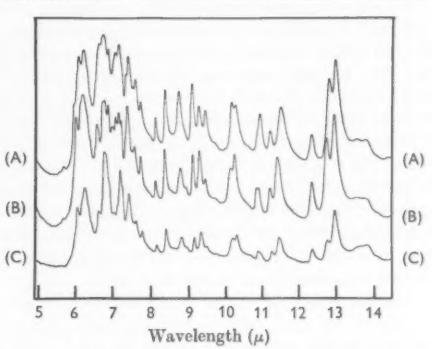


Fig. 1. Infrared spectra of β-pyrazol-1-ylalanine. (A) Natural (−) form; (B) racemized natural; (C) synthetic (racemic). All spectra were determined with liquid-paraffin mulls of the amino acid.

SUMMARY

1. A heterocyclic amino acid, (−)-β-pyrazol-1-ylalanine, was isolated from the seed of water melon (*Citrullus vulgaris*) and its structure confirmed by comparison with synthetic material.

2. DL-β-Pyrazol-1-ylalanine was synthesized in poor yield by condensation of silver pyrazole with the methyl ester of β-chloroalanine hydrochloride.

3. The amino acid was decomposed by treatment with 55 % (w/w) hydriodic acid to give alanine and a substance tentatively identified as an iodopyrazole.

4. The distribution and possible method of biogenesis of the amino acid were examined briefly.

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REFERENCES

Bryant, M. & Fowden, L. (1959). *Ann. Bot., Lond.*, N.S. **23**, 65.
Finar, I. L. & Utting, K. (1960). *J. chem. Soc.* (in the Press).
Fischer, E. & Raske, K. (1907). *Ber. dtsch. chem. Ges.* **40**, 3719.
Fowden, L. (1958). *Biol. Rev.* **33**, 393.
Fowden, L. (1960). *Rep. Progr. Chem.* **56**, 359.
Fowden, L. & Webb, J. A. (1958). *Ann. Bot., Lond.*, N.S. **22**, 73.
Gmelin, R. (1959). *Hoppe-Seyl. Z.* **316**, 164.
Greenberg, J. B. & Galston, A. W. (1959). *Plant Physiol.* **34**, 489.
Hattori, S. & Komamine, A. (1959). *Nature, Lond.*, **183**, 1116.
Hunt, G. E. (1959). *Bot. Rev.* **25**, 148.
Jacobs, T. L. (1957). In *Heterocyclic Compounds*, vol. 5, p. 48. Ed. by Elderfield, R. C. New York: John Wiley and Sons Inc.
Jepson, J. B. & Smith, I. (1953*a*). *Nature, Lond.*, **171**, 43.
Jepson, J. B. & Smith, I. (1953*b*). *Nature, Lond.*, **172**, 1100.
Mudd, J. B. & Zalik, S. (1958). *Canad. J. Bot.* **36**, 467.
Noe, F. F. & Fowden, L. (1959). *Nature, Lond.*, **184**, 69.
Partridge, S. M. (1949). *Biochem. J.* **44**, 521.
Polyanovskii, O. L. & Kretovich, V. L. (1957). *Dokl. Akad. Nauk, S.S.S.R.* **112**, 1086.
Schneider, G. (1958). *Biochem. Z.* **330**, 428.
Shinano, S. & Kaya, T. (1957). *J. agric. chem. Soc. Japan*, **31**, 759.
Virtanen, A. I. (1957). *Festschrift Arthur Stoll*, p. 565. Basel: Sandoz.

APPENDIX

Nuclear Magnetic Resonance Spectrum of β-Pyrazol-1-ylalanine

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The results described above indicated that β-pyrazol-1-ylalanine probably contained an alanine side chain attached to a pyrazole or imidazole ring. The nuclear magnetic resonance spectrum of the amino acid was therefore compared with spectra of pyrazole, imidazole and some of their derivatives in order to identify the ring and to determine the position of attachment of the side chain. Hydrogen nuclear magnetic resonance spectra were obtained with a Varian V 4300B high-resolution spectrometer operating at 40 Mcyc. Compounds were examined as solutions in deuterium oxide or sodium deuteroxide in deuterium oxide contained in sample tubes of 5 mm. diam. Spectra were calibrated by the side-band technique, with a capillary tube containing water as an external reference.

The characteristics of the spectra due to the hydrogen nuclei in imidazole and pyrazole ring systems are summarized in Table 1. The spectrum of imidazole consists of two peaks which show no fine structure. The separation between the peaks is 25 cyc./sec., the line of greater area being closer to the reference line. Substitution at the nitrogen atom does not produce any major change in the part of the spectrum due to the hydrogen atoms attached to the heterocyclic ring. In the spectrum of pyrazole, the two peaks both show fine structure, the smaller peak being a triplet and the larger a doublet. The separation between the two peaks is 52 cyc./sec., the peak of smaller area being closer to the reference line. Again, substitution at the nitrogen atom does not produce any marked change in the spectrum of the ring hydrogen atoms. As only small quantities of β-pyrazol-1-ylalanine were available, it was not possible to achieve the highest resolution in the spectrum, and the fine structure was not fully resolved. The spectrum of the heterocyclic hydrogen atoms of the new amino acid is summarized in Table 1.

Table 1. *Nuclear magnetic resonance spectra of hydrogen atoms in imidazole- and pyrazole-ring systems*

Compound	Shift to low field with respect to reference line* (cyc./sec.)	Separation of peaks (cyc./sec.)	Multiplet structure	Relative areas	Assignment (position)
Imidazole	73	25	Singlet	2	4:5
	98		Singlet	1	2
N-Methylimidazole	74	21	Singlet	2	4:5
	95		Singlet	1	2
Pyrazole	46	52	Triplet	1	4
	98		Doublet	2	3:5
N-Methylpyrazole	46	49	Triplet	1	4
	95		Doublet	2	3:5
Isolated material†	64	49	Triplet	1	—
	113		(Unresolved doublet?)	2	—

* Where the peaks show fine structure, this shift refers to the centre of the multiplet.
† Examined as a solution in 3M-NaOD in D_2O; the other compounds were studied in D_2O alone.

The fact that two lines of relative areas 1:2 are obtained shows that any substitution in the heterocyclic ring must have occurred at the nitrogen atom. In a *C*-substituted molecule, the spectrum would consist of either a single line or of two lines of equal areas. The separation of 49 cyc./sec. between the peaks, the fine structure and the relative positions of the peaks (the peak of smaller area being closer to the reference line) all indicate that the heterocyclic system is a pyrazole ring and not an imidazole ring. The remainder of the spectrum, which consists of a doublet and triplet in the region characteristic of hydrogen atoms attached to saturated carbon atoms, indicates the presence of a $-CH_2-CH\langle$ group; this is consistent with the presence of an alanine side chain attached to the heterocyclic nitrogen atom.

The spectrum of synthetic β-pyrazol-1-ylalanine was identical with that of the isolated material.

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Frequencies of Nucleotide Sequences in Deoxyribonucleic Acids

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Burton & Petersen (1957, 1960) have studied the degradation of deoxyribonucleic acid from calf thymus by the action of diphenylamine in aqueous formic acid at 30°. The products include a complex mixture of pyrimidine nucleotides that represent the sequences of pyrimidine nucleotides which were originally attached at each end to a purine nucleotide. Several of these nucleotides can be separated by paper chromatography after treatment with phosphomonoesterase. Since the degradation and enzymic dephosphorylation appear to go to completion without complicated side reactions or exchange processes these procedures constitute a simple method for determining the frequencies of a number of sequences in the deoxyribonucleic acid. This paper describes additional results obtained with calf-thymus deoxyribonucleic acid as well as with preparations of deoxyribonucleic acid from four species of bacteria, from herring testes and from the sperm of salmon and of a sea urchin (*Echinus esculentus*). Some of the results have been communicated to The Biochemical Society (Burton, 1960).

MATERIALS AND METHODS

Nucleases. Pancreatic deoxyribonuclease and ribonuclease were obtained from Sigma Chemical Co., St Louis 18, Mo., U.S.A.

Deoxyribonucleic acids. A preparation from calf thymus was obtained by virtually the same procedure as that described by Burton & Petersen (1960). Salmon-sperm

deoxyribonucleic acid (DNA) was purchased from California Corporation for Biochemical Research, Los Angeles 63, Calif., U.S.A. It had been prepared according to Emanuel & Chaikoff (1953) but with certain unspecified modifications. Herring-testes DNA was prepared according to Emanuel & Chaikoff (1953).

Sperm of *Echinus esculentus* were mixed with 2 vol. of ethanol for transport to the laboratory. With the aid of a blender, the insoluble material was suspended in 2·5 l. of 2M-NaCl containing 5mM-disodium ethylenediaminetetraacetic acid. The suspension was filtered through a thin layer of Celite and the filtrate was treated with NaCl and then filtered according to Emanuel & Chaikoff (1953). The filtrate thus obtained was treated with chloroform in a blender and the resulting denatured protein was removed by centrifuging at 15 000 *g*. The DNA was precipitated by ethanol. To remove NaCl, the DNA was dissolved in water at 0° and reprecipitated with ethanol, and the solution and precipitation were repeated.

DNA was prepared from *Escherichia coli* according to Burton (1956), except that the treatment of the bacteria with sodium dodecyl sulphate was omitted.

Strain C10 of *Pseudomonas aeruginosa* (Dickinson & Codd, 1952) was grown overnight in a medium of 0·8% of tryptone, 0·5% of NaCl and 1% of glucose at 30° with aeration. The cells from 16 l. were treated in a blender with 200 ml. of 0·5M-NaCl containing 0·25% of sodium dodecyl sulphate in order to remove any extracellular DNA. (Later experiments with pancreatic deoxyribonuclease showed that the amounts of extracellular DNA were negligible in similar cultures.) The mixture was centrifuged, the cells were broken in a press (Hughes, 1951), suspended in 500 ml. of M-NaCl and treated in a blender with 50 ml. of chloroform for about 5 min. After centrifuging, the precipitate was suspended in M-NaCl and the treatment in the blender was repeated. The supernatants from the two treatments in the blender were combined and mixed with 1·5 vol. of ethanol. The resulting precipitate was dissolved in water, reprecipitated with ethanol and treated again in the blender with M-NaCl and chloroform. After centrifuging, the supernatant was mixed with ethanol and the resulting fibrous precipitate was suspended in 100 ml. of 0·07M-triethanolamine buffer, pH 7·4. Most of the insoluble material was removed by high-speed centrifuging and the supernatant was incubated at 40° for 1 hr. with 1 mg. of five-times crystallized ribonuclease. After agitation with chloroform, centrifuging and precipitation with ethanol, the DNA was dialysed, first against 2M-NaCl and then against two lots of 20 vol. of water. The material was clarified by centrifuging and the DNA was precipitated by ethanol and dried over $CaCl_2$.

A culture of *Alcaligenes faecalis* (N.C.T.C. 1347) was grown overnight in a medium of 1% of peptone, 1% of tryptone and 0·5% of yeast extract with aeration at 30°. The cells from 10 l. were treated with crystalline deoxyribonuclease to remove any extracellular DNA and then they were washed twice with water and broken in a press (Hughes, 1951). The material was treated in a blender for about 5 min. with 50 ml. of chloroform and 180 ml. of ice-cold M-NaCl containing 0·5% of sodium dodecyl sulphate. The precipitate obtained on centrifuging was then extracted twice by stirring with 300 ml. of 0·5M-NaCl. The extracts were combined with the supernatants obtained after the two treatments in the blender and nucleic acids were then precipitated by ethanol. They were treated with ribonuclease and the DNA was isolated in the same way as with the material from *P. aeruginosa*.

A sample of DNA from *Micrococcus lysodeikticus* strain 53–20 was very kindly given by Dr H. B. Naylor (cf. Scaletti & Naylor, 1959).

Purity of deoxyribonucleic acids. Except for the salmon DNA, which contained 8·3% of P, the samples contained only 5·0–7·5% of P and were therefore not pure. Traces of protein were present in most of the samples but the following evidence indicates that less than 2% of the P was present as ribonucleic acid. The mixtures obtained by degradation of the DNA samples with diphenylamine and removal of the reagents by ether were found to react in the orcinol reaction as if they contained 5–6 moles of ribose per 100 g. atoms of P. The procedure of Ogur & Rosen (1950) was followed. Most of this apparent ribose was presumably due to the pyrimidine deoxyribonucleotides, since the conditions are drastic enough to hydrolyse their glycosidic links and since deoxyribose itself or each of the four common deoxyribonucleosides (California Corporation for Biochemical Research, Los Angeles 63, Calif., U.S.A.) reacted as if they contained 8–10 moles of ribose/100 moles of deoxyribose.

As mentioned in the Discussion, the results obtained with the DNA from *M. lysodeikticus* can be taken to suggest that the material may have contained 10–20% of the P as an unidentified impurity.

Table 1. *Base compositions*

Amounts are given as moles/100 moles of the four bases ±S.E.M. The values for *A. faecalis* DNA are those given by Spirin, Belozersky, Shugayeva & Vanyushin (1957) and by Catlin & Cunningham (1958) respectively. The figures in parentheses indicate the number of values.

DNA	Adenine	Guanine	Cytosine	Thymine	$\frac{\text{Adenine + thymine}}{\text{Guanine + cytosine}}$	$\frac{\text{Thymine}}{\text{Cytosine}}$
M. lysodeikticus	13·1	33·0	39·6	14·2	0·38	0·30
A. faecalis	16·5 15·9	33·9 31·5	32·9 36	16·8 16·2	0·49±0·01 (range)	0·48±0·03 (range)
P. aeruginosa	16·0±0·2 (9)	29·6±0·6 (8)	36·3±0·3 (9)	17·8±0·6 (9)	0·50±0·03	0·49±0·02
Escherichia coli	26·8±0·6 (4)	23·2±0·4 (4)	25 ±0·4 (4)	25 ±0·4 (4)	1·08±0·04	1·0 ±0·03
Salmon	27·8±0·4 (5)	22·7±0·3 (4)	26·5±0·2 (8)	28 ±0·4 (8)	1·26±0·02	1·30±0·02
Calf	28·6±0·3 (11)	21·2±0·4 (11)	21·7±0·4 (13)	28·5±0·6 (13)	1·33±0·02	1·32±0·04
Herring	31 ±0·4 (5)	20 ±0·4 (5)	21 ±0·05 (5)	28 ±0·6 (5)	1·44±0·04	1·40±0·02
Echinus esculentus	31·6±0·4 (8)	18 ±0·6 (8)	19·2±0·3 (8)	31·6±0·7 (8)	1·70±0·03	1·65±0·05

Base analyses. These were performed by hydrolysis in 7·5N-perchloric acid and paper chromatography with propan-2-ol–conc. HCl–water (65:20:10, by vol.; Wyatt, 1955). The values found are shown in Table 1. In all the analyses, between 90 and 100 moles of base were recovered per 100 g.atoms of P. The results agree well with the appropriate literature values (Chargaff, 1955; Lee, Wahl & Barbu, 1956; Spirin, Belozersky, Shugayeva & Vanyushin, 1957), although there are some small differences for the herring, *Echinus* and *Pseudomonas* preparations of DNA. The values found for salmon DNA are slightly different from those given by Chargaff (1955) but they agree very closely with those of Emanuel & Chaikoff (1958).

There was not enough of the DNA from *A. faecalis* or *M. lysodeikticus* to permit measurement of the base compositions. The values shown for *A. faecalis* are those given by Spirin *et al.* (1957) and by Catlin & Cunningham (1958). The values for *M. lysodeikticus* are those given by Scaletti & Naylor (1959) for the same strain.

Nomenclature. As in the previous paper (Burton & Petersen, 1960), C and T represent deoxycytidine and thymidine respectively and p represents orthophosphate esterified with these deoxynucleosides. A p symbol to the left of a nucleoside symbol is esterified to the 5′ position. A p symbol to the right of a nucleoside symbol is at the 3′ position, except in empirical formulae such as CTp or CT_2p_2, which do not indicate the sequence of nucleosides or the position(s) of the phosphate groups.

RESULTS

Following the procedures of Burton & Petersen (1960), the eight samples of DNA were degraded with diphenylamine in 66% (v/v) formic acid, the amine and formic acid were removed by ether extraction and the purines were removed by Dowex-50 cation-exchange resin in the H^+-ion form. The pyrimidine nucleotides were treated with purified phosphomonoesterase until no more inorganic phosphate was released and the amounts of the various nucleotides were determined after separation by two-dimensional chromatography on paper. It was desirable to cut out the thymidine area before

Table 2. *Amounts of inorganic phosphate after diphenylamine treatment and after subsequent treatment with phosphomonoesterase*

Figures in parentheses are the number of measurements obtained. Each measurement was performed in duplicate or triplicate.

DNA	Inorganic P (% of total P ± S.E.M.) After diphenylamine	After phosphatase
M. lysodeikticus	22, 21	71, 75
A. faecalis	24·5	75
P. aeruginosa	24·5, 23·5	75, 76
Escherichia coli	25·7 ± 0·4 (3)	71 ± 1·2 (3)
Salmon	25·2, 24·9	72·5
Calf	27·8 ± 0·4 (4)	72·2 ± 1·5 (3)
Herring	25·1 ± 0·5 (5)	72
Echinus esculentus	24·6 ± 0·6 (4)	73 ± 2 (3)

Table 3. *Amounts of nucleotides after treatments with diphenylamine and phosphatase*

The amounts were determined from the ultraviolet absorption of the eluates from two-dimensional paper chromatograms (Burton & Petersen, 1960). The number of eluates measured are shown in parentheses and the mean amounts ± S.E.M. are given in moles of pyrimidine/100 g.atoms of P on the chromatograms.

	Source of DNA: *M. lysodeikticus*	*A. faecalis*	*P. aeruginosa*	*Escherichia coli*	Salmon	Calf	Herring	*Echinus esculentus*
T	2·96 ± 0·1 (8)	5·5 ± 0·08 (6)	3·9 ± 0·05 (9)	5·3 ± 0·06 (6)	6·6 ± 0·19 (4)	6·53 ± 0·16 (8)	8·6 ± 0·15 (4)	8·4 ± 0·10 (4)
T_2p	0·22 ± 0·04 (5)	1·52 ± 0·08 (8)	1·15 ± 0·04 (11)	2·82 ± 0·06 (15)	3·15 ± 0·13 (5)	2·60 ± 0·06 (14)	3·1 ± 0·05 (5)	4·3 ± 0·30 (4)
T_3p_2		0·5 ± 0·05 (6)		0·96 ± 0·05 (11)	1·23 ± 0·04 (5)	1·21 ± 0·05 (14)	1·5 ± 0·1 (6)	2·14 ± 0·06 (5)
T_4p_3		0·1 (1)		0·32 ± 0·04 (10)	0·54 ± 0·04 (4)	0·57 ± 0·08 (11)	0·55 ± 0·03 (5)	0·90 ± 0·03 (4)
T_5p_4				0·14 ± 0·02 (5)	—	0·20 ± 0·04 (4)	—	0·3 (1)
C	6·9 ± 0·09 (8)	8·3 ± 0·3 (8)	8·9 ± 0·13 (10)	6·3 ± 0·12 (14)	4·05 ± 0·12 (6)	3·85 ± 0·06 (14)	5·9 ± 0·14 (4)	5·1 ± 0·09 (5)
C_2p	4·9 ± 0·18 (9)	5·08 ± 0·06 (8)	7·1 ± 0·08 (8)	3·83 ± 0·1 (10)	1·71 ± 0·18 (4)	1·77 ± 0·05 (12)	1·8 ± 0·06 (5)	1·9 ± 0·04 (5)
C_3p_2	1·7 ± 0·11 (9)	1·4 ± 0·1 (8)	1·4 ± 0·23 (9)	0·97 ± 0·04 (10)	0·44 (1)	0·77 ± 0·05 (7)	0·64 ± 0·03 (5)	0·5 ± 0·02 (3)
CpT	1·45 ± 0·03 (8)	3·5 ± 0·15 (6)	3·25 ± 0·1 (10)	2·78 ± 0·08 (12)	3·56 ± 0·15 (6)	3·04 ± 0·06 (11)	3·2 ± 0·05 (4)	2·3 ± 0·09 (3)
TpC	2·5 ± 0·07 (8)	2·28 ± 0·03 (6)	3·9 ± 0·05 (10)	3·15 ± 0·07 (12)	2·11 ± 0·13 (6)	2·21 ± 0·05 (11)	2·0 ± 0·01 (5)	2·9 ± 0·07 (3)
CT_2p_2	0·96 ± 0·04 (8)	2·65 ± 0·11 (8)	1·9 ± 0·08 (12)	2·20 ± 0·07 (13)	2·80 ± 0·20 (5)	2·73 ± 0·11 (10)	2·8 ± 0·09 (5)	3·1 ± 0·05 (5)
CT_3p_3		1·1 ± 0·1 (7)	0·5 ± 0·04 (9)	1·14 ± 0·04 (11)	1·24 ± 0·09 (5)	1·43 ± 0·07 (7)	0·98 ± 0·07 (4)	1·7 ± 0·09 (5)
CT_4p_4		0·5 ± 0·1 (4)	— —	0·50 ± 0·02 (6)	0·7, 0·96 (2)	0·92 ± 0·11 (3)	0·70 ± 0·04 (4)	1·1, 1·1 (2)
CT_5p_5		— —	— —	— —	—	0·49 (1)	0·8 (1)	—
C_2Tp_2	3·15 ± 0·03 (9)	3·4 ± 0·08 (8)	4·6 ± 0·1 (8)	2·34 ± 0·10 (6)	3·11 ± 0·15 (4)	2·90 ± 0·11 (4)	2·3 ± 0·10 (5)	2·0 ± 0·10 (4)
C_2Tp_2p	1·0 ± 0·10 (5)	2·1 ± 0·2 (5)	2·6 ± 0·13 (8)	1·13 ± 0·04 (6)	2·25, 1·42 (2)	1·63 ± 0·17 (3)	1·6 ± 0·17 (5)	1·3, 1·1 (2)
C_2Tp_3	—	—	0·97 (1)	— —	— —	— —	1·05 ± 0·10 (3)	1·8 (1)
C_3Tp_3	1·5 ± 0·21 (6)	— —	2·6, 2·8 (2)	— —	— —	— —	1·07, 1·1 (2)	— —

development with the 2-methylpropan-2-ol–HCl–water mixture because otherwise it tended to become contaminated with impurities which absorbed ultraviolet light.

The amounts of phosphate released before and after the phosphatase treatment are shown in Table 2 and the amounts of the various nucleotides are shown in Table 3. Except for the salmon and *A. faecalis* preparations of DNA, the results in Table 3 were obtained from two or three different degradations of each DNA. The results obtained with different portions of the same DNA were in good agreement with each other.

DISCUSSION

Some of the results are presented diagrammatically in Fig. 1 and compared with the amounts of nucleotides which should have been found for randomly arranged polynucleotides of the same thymine/cytosine ratios. More detailed comparisons are shown in Table 4. The expected amounts were calculated according to Jones, Stacey & Watson (1957), and it was assumed that the thymine and cytosine together represented 50 % of the bases. Each DNA differs markedly from each other and from the randomly arranged polynucleotide, and there is no obviously consistent pattern. Although the distributions reflect the variations in thymine/cytosine ratios there are considerable differences between the amounts of the nucleotides in DNA preparations of closely similar ratios. For all the DNA preparations except *A. faecalis*, T_2p and CT_2p_2 are obtained in amounts which are less than the random value.

Isomeric dinucleotides. The two sequential isomers CpT and TpC were separated in the two-dimensional chromatograms and, as shown in Fig. 2, the relative amounts differ for the various DNA preparations. The ratio shows no relation to the base composition and ranges from 0·6 for *M. lysodeikticus* to 1·7 for salmon DNA. It is clearly desirable to attempt to determine the proportion of isomers in the tri- and tetra-nucleotide components.

Bunching of pyrimidines and purines. Tamm, Shapiro, Lipshitz & Chargaff (1953) and Shapiro & Chargaff (1957, 1960) have claimed that there is a pronounced tendency for the nucleotides to be arranged in blocks of pyrimidines followed by blocks of purines. On the basis of the present results, such a tendency can be conveniently assessed in two somewhat independent ways: (i) from the amounts of inorganic phosphate; (ii) from the amounts of mono- and di-pyrimidine fragments.

The inorganic phosphate released by the action of diphenylamine was originally present as phosphodiester bridges between consecutive purine nucleosides. A randomly arranged polynucleotide with equimolar amounts of purines and pyrimidines would contain 25 % of its phosphate in the interpurine positions. The results in Table 2 therefore show that there is no general tendency for purines to be bunched together, since it is only in calf-thymus DNA that appreciably more than 25 % of the phosphate is released by diphenylamine.

Although the amounts of interpurine phosphate agree within analytical error with the amounts of organic phosphate remaining after the phosphatase treatment, it is not possible exactly to equate amounts of the latter with the interpyrimidine phosphate because the DNA samples could have contained up to 2 % of ribonucleic acid as well as other phosphorus compounds. Indeed the agreements found can perhaps be taken as evidence to

Table 4. *Comparison (on percentage basis) of amounts of nucleotides found with those expected for randomly arranged polynucleotides of the same base composition*

The deviations are relatively larger than those in Table 3. They were calculated by the formulae $100(f/\phi)\sqrt{(s_1^2/f^2 + s_2^2/\phi^2)}$, where $f \pm s_1$ is the observed frequency and S.E.M. of the nucleotides (Table 3). ϕ is the frequency calculated for a randomly arranged polynucleotide and s_2 is the S.E. introduced by the base analyses.

Source of DNA ...	*A. faecalis*	*P. aeruginosa*	*Escherichia coli*	Salmon	Calf	Herring	*Echinus esculentus*
T	136± 6	94± 4	85± 3	93± 4	91± 4	118±3	107± 4
T_2p	112±11	85± 8	90± 6	81± 5	65± 2	73±3	89± 8
T_3p_2	146±20		82± 9	73± 5	70± 2	81±7	95± 9
T_4p_3	—	—	82±10	85±10	88±16	77±6	99±13
C	99± 6	106± 4	101± 4	75± 4	72± 3	113±3	93± 3
C_2p	89± 8	128±11	123± 9	73± 9	77± 6	83±4	107± 7
C_3p_2	49± 7	50±10	83± 8	—	103±12	94±7	100±10
CpT	128± 6	118± 4	89± 3	116± 5	100± 3	105±2	78± 3
TpC	83± 1	142± 3	101± 3	69± 5	72± 2	66±1	99± 3
CT_2p_2	133± 9	93± 6	62± 4	71± 6	69± 4	70±1	76± 4
CT_3p_3	185±24	83±10	73± 6	64± 6	75± 6	48±5	75± 7
CT_4p_4	—	—	81± 9	—	102±16	74±8	—
C_2Tp_2	82± 5	111± 6	66± 4	103± 6	98± 6	80±4	80± 6
$C_2T_2p_3$	113±11	138± 8	48± 3	—	72± 8	72±8	—

suggest that any such impurities were present in only small amounts except in the DNA from *M. lysodeikticus*.

The second way of assessing the degree of bunching is to examine whether the amounts of pyrimidines in the mono- and di-pyrimidine fragments are less than the amount which is expected for a randomly arranged polynucleotide, namely 50 % of the total pyrimidines. The values found are 38 % for the DNA of *M. lysodeikticus*, 40 % for calf-thymus DNA, 42·6 % for salmon DNA and between 48 and 56 % for the other five DNA preparations.

Thus both assessments indicate that there is in calf-thymus DNA a slight but definite tendency for bunching of nucleotides into blocks of pyrimidines and blocks of purines. In salmon DNA there seems to be a slight bias against mono- and di-pyrimidine runs, although the total amount of interpurine phosphate is still 25 %. Since five of the other six DNA preparations show no appreciable tendency towards such a bunching of nucleotides, it can be concluded that this is not a general property of DNA. Even with calf-thymus DNA, the degree of bunching appears to be much less than that which was claimed by Tamm *et al.* (1953) and by Shapiro & Chargaff (1957).

The results obtained with *M. lysodeikticus* DNA are somewhat contradictory in suggesting a strong tendency against bunching by the first assessment and a strong tendency towards bunching by the second. They could be more easily understood if 10–20 % of its phosphate were present as an unidentified impurity which is incompletely dephosphorylated by the phosphatase. A correction of 15 % has therefore been arbitrarily applied to the values shown for this DNA in Figs. 1 and 2. The figures in Table 1 have not been corrected in this way and values for this DNA are not included in Table 4.

Josse & Kornberg (1960; and personal communication) have used the deoxyribonucleotide-polymerase system to study the frequencies of the sixteen kinds of consecutive pairs of nucleotides in the primer DNA. Their results agree with those given here in showing that there is a slight tendency

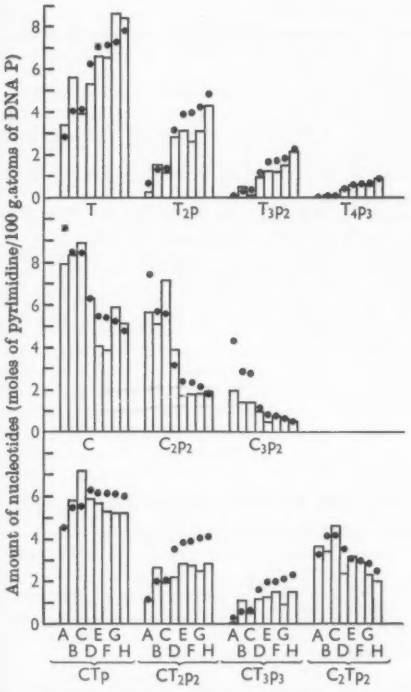

Fig. 1. Amounts of nucleotides obtained. For each nucleotide, the eight rectangles indicate the amounts for the respective DNA samples: A, *Micrococcus lysodeikticus*; B, *Alcaligenes faecalis*; C, *Pseudomonas aeruginosa*; D, *Escherichia coli*; E, salmon sperm; F, calf thymus; G, herring testes; H, sea-urchin sperm (*Echinus esculentus*). For reasons given in the Discussion, the amounts for A as given in Table 3 have been increased by 15 %. ●, Amounts expected for randomly arranged polynucleotides of the same base composition as the corresponding DNA. CTp, Sum of the two isomers CpT and TpC.

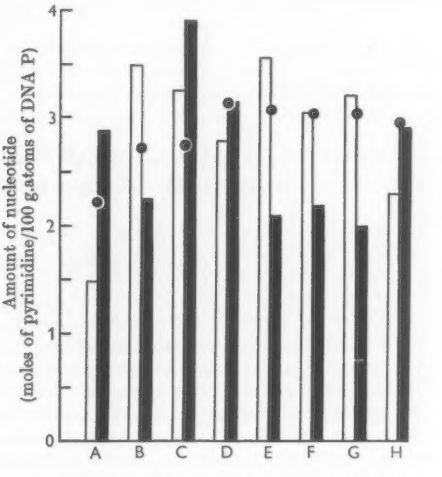

Fig. 2. Amounts of the isomers, deoxycytidylyl-(3′ → 5′)-thymidine (CpT) and thymidylyl-(3′ → 5′)-deoxycytidine (TpC). □, CpT; ■, TpC; ●, random values. DNA samples A–H are as identified in Fig. 1. The values for *M. lysodeikticus* from Table 3 have been increased by 15 %.

for purines to be bunched in calf-thymus DNA and essentially no tendency in the other five types of DNA which were examined. Since these five sources include *M. lysodeikticus*, their results are evidence for the validity of the correction of 15% which has been applied to the results for this DNA in Figs. 1 and 2.

SUMMARY

1. Preparations of deoxyribonucleic acid (DNA) from eight sources (four bacterial species and four animal species) have been degraded with diphenylamine in formic acid. The amounts of inorganic phosphate and of several pyrimidine nucleotides have been measured.

2. The results have been compared with those expected for randomly arranged polynucleotides of the same base composition. Each DNA differs from the others and from the randomly arranged polynucleotide but there is no obviously consistent pattern. There appears to be a tendency for T_2p and CT_2p_2 to be obtained in amounts which are less than the random value.

3. There is no general tendency for purines or pyrimidines to be bunched together in a non-random fashion.

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REFERENCES

Burton, K. (1956). *Biochem. J.* **62**, 315.
Burton, K. (1960). *Biochem. J.* **74**, 35P.
Burton, K. & Petersen, G. B. (1957). *Biochim. biophys. Acta*, **26**, 667.
Burton, K. & Petersen, G. B. (1960). *Biochem. J.* **75**, 17.
Catlin, B. W. & Cunningham, L. S. (1958). *J. gen. Microbiol.* **19**, 522.
Chargaff, E. (1955). In *The Nucleic Acids*, vol. 1, p. 307. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
Dickinson, L. & Codd, S. (1952). *J. gen. Microbiol.* **6**, 1.
Emanuel, C. F. & Chaikoff, I. L. (1953). *J. biol. Chem.* **203**, 167.
Emanuel, C. F. & Chaikoff, I. L. (1958). *Biochim. biophys. Acta*, **28**, 550.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
Jones, A. S., Stacey, M. & Watson, B. E. (1957). *J. chem. Soc.* p. 2454.
Josse, J. & Kornberg, A. (1960). *Fed. Proc.* **19**, 305.
Lee, K. Y., Wahl, R. & Barbu, E. (1956). *Ann. Inst. Pasteur*, **91**, 212.
Ogur, M. & Rosen, G. (1950). *Arch. Biochem.* **25**, 262.
Scaletti, J. V. & Naylor, H. B. (1959). *J. Bact.* **78**, 422.
Shapiro, H. S. & Chargaff, E. (1957). *Biochim. biophys. Acta*, **26**, 608.
Shapiro, H. S. & Chargaff, E. (1960). *Biochim. biophys. Acta*, **39**, 68.
Spirin, A. S., Belozersky, A. N., Shugayeva, N. Y. & Vanyushin, B. F. (1957). *Biokhimiya*, **22**, 744.
Tamm, C., Shapiro, H. S., Lipshitz, R. & Chargaff, E. (1953). *J. biol. Chem.* **203**, 673.
Wyatt, G. R. (1955). In *The Nucleic Acids*, vol. 1, p. 243. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.

Biochem. J. (1960) **77**, 552

Levels and Distribution of the Acid-Soluble Ribonucleotides in the Mammary Glands of Pregnant and Lactating Rats

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The striking increases in the rate of metabolic and synthetic activity which are known to occur in the mammary gland in the transition from the relative quiescence of late pregnancy to the fully active state obtaining in late lactation could be expected to be reflected in similar changes occurring in either the level or turnover (or both) of the acid-soluble ribonucleotides.

Some studies on the distribution of the acid-soluble ribonucleotides in the mammary gland have already been reported. For instance, Forrest, Wilken & Hansen (1960) have examined the nucleotide levels in rat mammary tissue at late lactation and Manson (1956) has made similar studies in the cow and goat. Both of these studies found a pattern of distribution essentially similar to that found in rat liver by Hurlbert, Schmitz, Brumm & Potter (1954). Unfortunately, these investigations covered only one stage of lactation and did not attempt to correlate the level of ribonucleotides with lactational activity. Further Denamur, Fauconneau & Guntz (1958) have

studied the distribution of the nucleotides in ewe colostrum and milk at different stages of the lactation cycle and reported large-scale changes. There are no data on the changes in the glandular tissue itself although the metabolic changes mentioned above and the large increases in both ribonucleic acid and deoxyribonucleic acid (Kirkham & Turner, 1953; Greenbaum & Slater, 1957*b*; but see also McLean, 1958*a*) of rat mammary gland would suggest that increases in the nucleotides might be expected.

The present study on the changes in the level of acid-soluble ribonucleotides of rat mammary gland in response to the increase in lactational activity shows that the mammary gland contains most of the nucleotides present in rat liver in approximately the same ratios and further that there is a general increase in most of the ribonucleotides to a value approximately three times that found in late pregnancy.

METHODS

Animals. Adult female albino rats undergoing their first pregnancy or lactation were used in these investigations. The number of pups was restricted to eight and all rats with litters of less than six were rejected. The length of pregnancy was ascertained from the mating date and in this colony averages 22 days.

Chromatography of ribonucleotides. Each experiment was performed on the combined abdominal mammary glands of one rat (weight approx. 4–5 g.) or, where larger quantities of material were required for the rechromatography of the peaks, the pooled abdominal glands of three animals. The rats were killed by dislocation of the cervical vertebrae and the lower abdominal glands were quickly removed and placed into liquid nitrogen. The frozen tissue was then weighed and ground to a powder in the frozen state and extracted with ice-cold perchloric acid as described by Hurlbert *et al.* (1954). The extracts were neutralized with 5N-KOH and the potassium perchlorate was removed by centrifuging. Analysis of the neutralized extracts by anion-exchange chromatography on Dowex-1 (formate form) columns was then carried out by the gradient-elution procedure of Hurlbert *et al.* (1954) with three changes of solvent. The mixing vessel (initially containing water) was of 500 ml. volume and the resin column was 10 cm. × 0·7 cm.^{2} in size. When pooled tissue samples were used the column length was increased to 15 cm. for better resolution. The effluent from the columns was collected in 5 ml. fractions and the extinction was measured at 260 mμ. The development of the chromatogram generally took 3 days and was carried out without interruption of the flow, as it was found that any interruption of the elution procedure produced spurious peaks and distortion of the chromatogram. When rechromatography of the peaks was required the appropriate fractions were freeze-dried to remove the water and formic acid. When the fractions contained ammonium formate this was removed by exposing the freeze-dried fractions to a higher vacuum, attained by the use of an oil-diffusion pump, and the flasks were cautiously warmed with infrared lamps as described by Hurlbert *et al.* (1954). Rechromatography was carried out by gradient elution with ammonium formate (Hurlbert *et al.* 1954). The mixing flask was of 250 ml. volume and the resin column was 10 cm. × 0·7 cm.^{2} With careful drying 80% of the u.v.-light-absorbing material could be recovered after rechromatography.

Identification of the ribonucleotides in the various peaks was based on the data of Hurlbert *et al.* (1954) and confirmed by the ratios of the extinction coefficients at 250, 260 and 280 mμ.

Chromatography of rat milk. Since the mammary glands as dissected from the animal contained a considerable volume of milk, the presence of high concentrations of ribonucleotides in this fluid could introduce an appreciable error into the estimation of the level of the nucleotides in the mammary tissue. A sample of milk was collected by milking out the glands of rats injected with 5 i.u. of Pitressin (Parke Davis and Co. Ltd.).

The acid-soluble ribonucleotides were determined in this milk and were found to be mainly uridine compounds. The total amount of nucleotide (based on a millimolar extinction coefficient at 260 mμ of 10) is of the order of 40 μmoles/100 ml. of milk. Assuming that 40% of the wet weight of the gland is due to the presence of retained milk in the tissue, an error of not more than 5% can be attributed to contamination of milk in the whole gland in the determination of total acid-soluble ribonucleotides.

RESULTS

Characterization of the individual fractions. Typical chromatograms for the nucleotides of the mammary glands from pregnant and lactating rats are shown in Figs. 1 and 2.

A series of fractions were isolated from each chromatogram as shown in these figures, and the compositions of these fractions were identified as follows.

I. This fraction consists of cytidine monophosphate and diphosphopyridine nucleotide (DPN). The cytidine monophosphate component was only about 10% in pregnancy, rising to 20% in lactation.

II. This fraction is composed solely of adenosine monophosphate (AMP). No other constituent was detectable.

III. This peak appears to consist almost entirely of guanosine monophosphate. No measureable triphosphopyridine nucleotide could be detected. This latter finding is in agreement with the results of McLean (1958*b*), who also failed to find any oxidized triphosphopyridine nucleotide in the mammary glands of rats.

IV. The fraction eluted at this position by Reid & Stevens (1957) with a liver extract was shown by them to contain cytidine diphosphate, inosine monophosphate and uridine monophosphate. Hurlbert *et al.* (1954) obtained these compounds as two peaks, one containing cytidine diphosphate and inosine monophosphate and the other uridine monophosphate. In the present study only one peak was observed and this seemed to be composed

entirely of uridine monophosphate both in pregnancy and lactation.

V. This fraction was pure adenosine diphosphate (ADP).

VI. This fraction was pure uridine diphosphate-*N*-acetylhexosamine, the $UDPX_1$ of Hurlbert *et al.* (1954). This substance was identified by the *p*-dimethylaminobenzaldehyde test of Aminoff, Morgan & Watkins (1952). This material has been tentatively identified as uridine diphosphate *N*-acetylglucosamine because it appeared in the same position in the chromatogram as an authentic sample of this compound. The tests, however, do not exclude the possibility of a greater or lesser contamination with the corresponding galactosamine compound.

VII. This fraction appears on the chromatogram and rechromatogram in the same position as uridine diphosphate glucose. On hydrolysis this peak was shown to contain a reducing sugar, as detected by the method of Park & Johnson (1949).

VIII. This fraction contained a single component identified as guanosine diphosphate.

IX. This fraction also contained only one component which corresponds to the compound ADX, described by Hurlbert *et al.* (1954).

X. The composition of this fraction was variable, dependent on the stage of lactation. In late pregnancy this fraction contained 75% of adenosine triphosphate (ATP), 25% of uridine diphosphate and no detectable uridine diphosphate hexuronic acid. In late lactation the composition approxi-

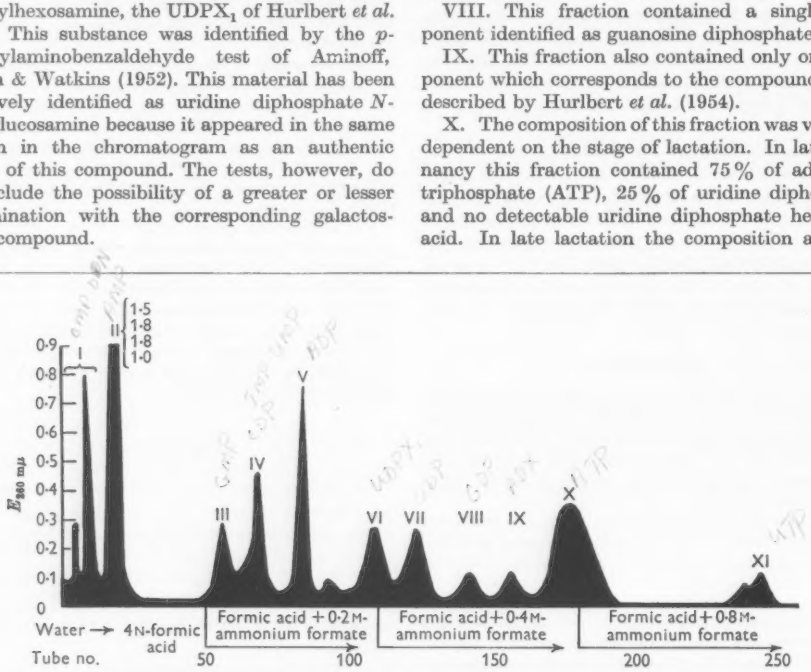

Fig. 1. Chromatogram of the acid-soluble ribonucleotides from the mammary tissue of one animal in late lactation. The method used Dowex-1 (formate form) ion-exchange resin and gradient elution with formic acid and ammonium formate as described in the Methods section. The number of 5 ml. fractions collected and the point of solvent change are indicated along the abscissae.

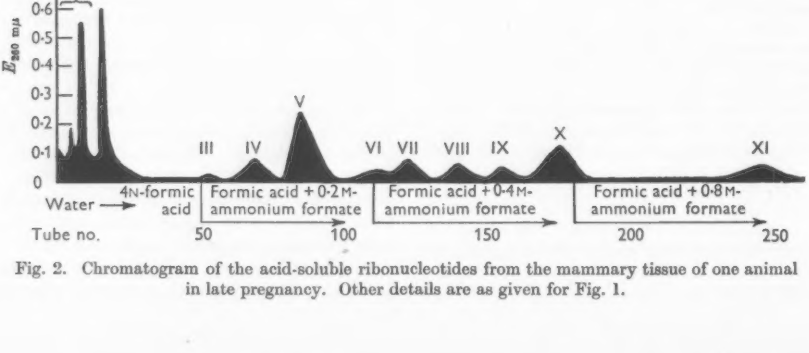

Fig. 2. Chromatogram of the acid-soluble ribonucleotides from the mammary tissue of one animal in late pregnancy. Other details are as given for Fig. 1.

mated to 65 % of ATP, 25 % of uridine diphosphate and 10 % of uridine diphosphate hexuronic acid. This last-named compound has been identified as uridine diphosphate hexuronic acid by virtue of its position on the rechromatogram and the positive test given for hexuronic acid when tested by the carbazole method of Dische (1947).

XI. On rechromatography this fraction could be resolved into three components in late lactation: uridine triphosphate (60 %), guanosine triphosphate (20 %) and a third component which appeared in the chromatogram in the same position as the unknown component cited by Hurlbert *et al.* (1954), and believed by them to be a pyrimidine nucleoside pyrophosphate. In pregnancy the amount of nucleotide was too low to permit a detection of this third component, if it were present, or even the amount of uridine triphosphate or guanosine triphosphate.

Variations in the concentration of the acid-soluble ribonucleotides during the lactation cycle. Because of the composite nature of some of the fractions in the chromatogram no attempt has been made to express the absolute amounts in Figs. 1 and 2 and the ordinate is the extinction at 260 mμ. For the results given in Table 1, which shows the tissue content of nucleotide of the mammary gland at times from late pregnancy to the end of lactation, molar units/100 g. of wet tissue are used. These figures were obtained from the known molar extinction coefficients and the determined composition of the complex fractions. There is a general increase in the concentration of the acid-soluble ribonucleotides during the passage from late pregnancy to late lactation (see also Table 1).

Variations in the concentration of the total ribonucleotide content are shown in Fig. 3 (*a*). It will be seen that there is a sharp rise in concentration immediately after parturition followed by a slow increase up to about the tenth day of lactation, where the level reaches a plateau value at about three times that obtaining in pregnancy.

Adenosine phosphates. Figs. 3 (*b*) and 3 (*c*) illustrate the changes in concentration of the adenosine phosphates AMP and ADP. (Changes in the ATP concentration are not given in this figure as

Table 1. *Distribution and variation of acid-soluble nucleotides at different stages of lactation*

Amounts are shown in μmoles/100 g. of tissue. Abbreviations used: DPN, diphosphopyridine nucleotide; CMP, cytidine monophosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate; UMP, uridine monophosphate; ADP, adenosine diphosphate; $UDPX_1$ and $UDPX_2$, *N*-acetylhexosamine and hexose derivatives of uridine diphosphate respectively; GDP, guanosine diphosphate; ADX, compound described by Hurlbert *et al.* (1954); ATP, adenosine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate.

	Molar absorption $\times 10^{-3}$ conversion factor											
Lactational state	18 DPN CMP	14·5 AMP	11·6 GMP	9·9 UMP	14·5 ADP	9·9 $UDPX_1$	9·9 $UDPX_2$	11·8 GDP	14·5 ADX	13* ATP etc.	10·9* GTP UTP	Total
Day of pregnancy												
17	5·6	11·0	Trace	10·1	13·8	Trace	Trace	Trace	Trace	10	Trace	50·5
17	7·8	13·1	Trace	18·2	13·1	4·0	6·1	5·1	4·1	23·1	4·6	94·6
17	7·8	13·1	—	—	—	—	—	—	—	—	—	—
18	5·6	11·7	Trace	10·1	15·9	Trace	Trace	Trace	Trace	13·9	3·7	40·9
18	6·2	12·1	Trace	10·5	14·3	Trace	Trace	Trace	Trace	15·4	6·3	68·1
19	9·5	12·4	Trace	11·1	19·3	Trace	Trace	7·6	6·2	33·1	11·0	110·2
Day of lactation												
0	9·5	14·5	Trace	20·2	21·4	Trace	Trace	Trace	Trace	21·6	8·3	95·5
0	7·7	13·1	5·2	10·1	9·0	Trace	Trace	Trace	Trace	13·9	7·4	65·9
1	11·2	23·4	12·9	28·2	37·9	14·1	12·1	2·5	2·8	27·7	10·5	183·3
5	27·8	29·7	7·8	21·2	34·5	—	—	—	—	48·5	17·4	—
7	10·6	13·1	6·9	14·1	20·7	12·1	13·1	4·2	3·4	33·1	11·9	143
8	13·3	60·0	13·8	49·5	39·3	21·2	27·3	7·6	6·9	28·5	10·1	278
11	8·9	35·2	14·7	34·3	34·5	—	—	—	—	—	—	—
11	8·3	40·0	17·2	31·3	31·1	23·2	33·3	10·1	13·1	32·3	12·8	253
14	12·8	57·3	20·7	39·4	36·6	—	—	—	—	—	—	—
14	13·9	49·7	24·1	48·5	35·9	19·2	39·4	13·5	11·1	30·7	30·2	316
17	10·0	23·5	25·8	41·4	38·6	21·2	33·3	9·3	12·4	35·4	43·1	293
18	13·9	52·4	11·2	30·3	24·2	23·2	20·2	7·6	5·5	19·2	6·4	214
18	15·6	69·0	19·8	54·5	59·3	40·4	45·5	14·4	11·1	41·5	13·8	385
19	13·9	46·9	15·5	42·4	42·8	28·3	34·3	11·9	9·0	23·1	9·2	277
19	10·6	49·0	19·8	38·4	42·8	26·3	27·3	6·8	6·2	23·8	9·2	260
20	7·8	46·9	14·7	31·3	35·2	26·3	37·4	10·2	8·3	40·8	12·8	272

* Calculated from the known molar extinction coefficients and the observed composition of the fraction.

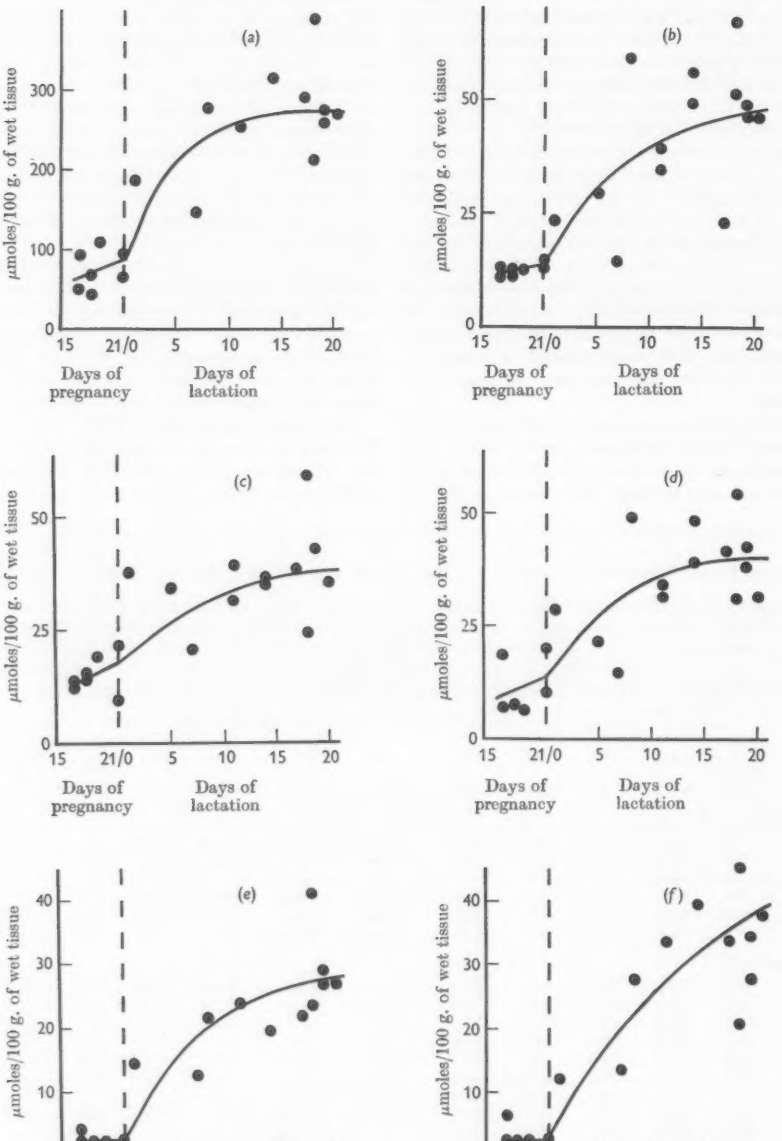

Fig. 3. Time curve of: (*a*) total acid-soluble nucleotides; (*b*) AMP; (*c*) ADP; (*d*) uridine monophosphate; (*e*) uridine diphosphate *N*-acetylhexosamine; (*f*) uridine diphosphate hexose.

these cannot be derived directly from the chromatograms, in which ATP appears as a composite peak.) Both of these nucleotides increase at parturition and at least the former continues to rise throughout lactation. The AMP curve shows a very sharp transition at parturition. It is worth noting that as lactation advances AMP increases in proportion to the other two adenine nucleotides (Table 1). The increase in ATP appears to be much less striking than that of AMP or ADP.

Uridine phosphates. Since the fractions containing uridine diphosphate, uridine triphosphate and uridine diphosphate hexuronic acid are composite peaks which are not easily resolved into the individual components the changes in these individual compounds with advancing lactation are not readily followed. Uridine diphosphate *N*-acetylhexosamine and uridine diphosphate hexose are clear-cut fractions and, as stated above, uridine monophosphate probably comprises the total nucleotides of the fraction in which it occurs. Thus figures for these three compounds are available and are shown in Figs. 3 (*d*), 3 (*e*) and 3 (*f*). There is considerable scatter in these figures of uridine diphosphate hexose (fraction VII) and uridine diphosphate *N*-acetylhexosamine (fraction VI), but both nevertheless show a pattern of a constant low value in pregnancy followed by a sharp rise at parturition and a slower rise during the course of lactation. Uridine monophosphate also has a low value in pregnancy and a sharp rise at parturition but there is only a small rise to a constant value which is attained about the tenth day of lactation.

Guanosine and cytidine phosphates. Both of these groups of phosphates increase over the lactation cycle (Table 1). Indeed, the guanosine and cytidine phosphates are present only in trace quantities during pregnancy. Although there is an increase in the concentrations of both these groups of compounds in late lactation the level attained is considerably lower than that of either the adenosine or uridine compounds.

DISCUSSION

The studies of Forrest *et al.* (1960) on rat, and Manson (1956) on cow and goat mammary-gland acid-soluble ribonucleotides relate to a single stage of lactation. The present study reports on changes in the level of these nucleotides at periods throughout the lactation cycle, during which there are considerable changes in the rate of protein and carbohydrate synthesis. It has been estimated (Brody & Nisbet, 1938) that the rate of protein synthesis increases tenfold over the period of lactation and it is probable that lactose synthesis increases by a similar amount. The observed threefold increase of the acid-soluble ribonucleotides may be correlated with two aspects of mammary-gland physiology: first the sevenfold rise in ribonucleic acid content of the gland (Slater & Planterose, 1958) and, secondly, the increase in the rate of synthetic activity. The ribonucleic acid content of the gland increases only up to the twelfth to fourteenth days of lactation and thereafter remains virtually constant (Greenbaum & Slater, 1957*b*, Shimizu, 1957), whereas the progress of synthetic activity, i.e. the rate of synthesis of milk, which may be deduced from the composition figures of rat milk (Cox & Mueller, 1937; Greenbaum & Slater, 1957*a*) and from the shape of the milk-yield curve given by Brody & Nisbet (1938), appears to increase linearly over the whole period of lactation. The curve showing the increase in total acid-soluble ribonucleotides (Fig. 3*a*) increases only up to about the tenth day of lactation and therefore shows a similar pattern of change to that found for the ribonucleic acid content of the gland.

Two rather surprising points emerge from the pattern of increase of the ribonucleotides. First there appears to be no correlation between the levels of nucleotide found and metabolic activity of the gland (as exemplified by the milk-yield curve or Q_{O_2} values of Folley & French, 1950). Secondly, it might have been expected that there would have been a preferential increase in adenosine phosphates, which would be associated with the increasing energy requirements of the gland and with the formation of the amino acid adenylates in protein synthesis, and of uridine compounds, which would be involved in the increased rate of carbohydrate transfer and in lactose synthesis. As can be seen from Table 1, there is no preferential increase in either of these two nucleotides. On the contrary all appear to increase together and to the same degree. It is probable that the activity of the nucleotides in terms of their coenzymic function would be better expressed as turnover rates and work is now in progress to measure these.

SUMMARY

1. The acid-soluble ribonucleotides of rat mammary gland have been measured at different stages of the lactation cycle. There is a general increase of these nucleotides so that the level of the total acid-soluble ribonucleotide is raised about threefold between late pregnancy and late lactation.

2. All the ribonucleotides appear to participate in this increase and all increase roughly to the same extent. Most of the nucleotides measured showed an abrupt increase at parturition and thereafter increased until about the fifteenth day of lactation, at which time they reached a plateau value.

3. The pattern of increase appears to be more closely related to the rate of increase of ribonucleic

acid in the gland than to the metabolic and synthetic activities of the tissue.

We wish to express our thanks to the Medical Research Council for the grant of a studentship to one of us (D.Y.W.) during the tenure of which this work was done.

REFERENCES

Aminoff, D., Morgan, W. T. J. & Watkins, M. W. (1952). *Biochem. J.* **51**, 379.
Brody, S. & Nisbet, R. (1938). *Res. Bull. Mo. agric. Exp. Sta.* no. 285.
Cox, W. M., jun. & Mueller, A. J. (1937). *J. Nutr.* **13**, 249.
Denamur, R., Fauconneau, G. & Guntz, G. (1958). *C.R. Acad. Sci., Paris*, **246**, 652.
Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
Folley, S. J. & French, T. H. (1950). *Biochem. J.* **46**, 465.
Forrest, R. J., Wilken, D. R. & Hansen, R. G. (1960). *Biochim. biophys. Acta*, **37**, 551.
Greenbaum, A. L. & Slater, T. F. (1957*a*). *Biochem. J.* **66**, 148.
Greenbaum, A. L. & Slater, T. F. (1957*b*). *Biochem. J.* **66**, 155.
Hurlbert, R. B., Schmitz, H., Brumm, A. F. & Potter, V. R. (1954). *J. biol. Chem.* **209**, 23.
Kirkham, W. R. & Turner, C. W. (1953). *Proc. Soc. exp. Biol., N.Y.*, **83**, 123.
McLean, P. (1958*a*). *Biochim. biophys. Acta*, **30**, 303.
McLean, P. (1958*b*). *Biochim. biophys. Acta*, **30**, 316.
Manson, W. (1956). *Biochim. biophys. Acta*, **19**, 398.
Park, J. T. & Johnson, M. J. (1949). *J. biol. Chem.* **181**, 149.
Reid, E. & Stevens, B. M. (1957). *Biochem. J.* **67**, 262.
Shimizu, H. (1957). *Tohoku J. agric. Res.* **7**, 339.
Slater, T. F. & Planterose, D. N. (1958). *Biochem. J.* **69**, 417.

Biochem. J. (1960) **77**, 558

Synthesis of Protein in a Purine-Requiring *Escherichia coli* Infected with Bacteriophage T2

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Strain B-96 is a mutant of *Escherichia coli* which requires adenine, guanine, hypoxanthine or xanthine for growth (Gots, 1950, 1953). Infection with T2r bacteriophage does not appear to alter the defect, since in the absence of adenine the deoxyribonucleic acid content of the culture remains constant and few bacteriophages are formed (Burton, 1955). This paper presents evidence that *E. coli* B-96 infected with T2r bacteriophage can, in the absence of exogenous adenine, synthesize bacteriophage-specific proteins and the enzymes required for the synthesis of bacteriophage deoxyribonucleic acid. The addition of adenine is, however, required for net synthesis of deoxyribonucleic acid.

METHODS AND MATERIALS

Bacteria. E. coli B-96 was obtained from Dr J. S. Gots. The bacteria were grown at 37° with aeration in the following growth medium (amounts/l.): 3·4 g. of Na_2HPO_4, 1·5 g. of KH_2PO_4, 1·0 g. of NH_4Cl, 6·0 g. of NaCl, 10 mg. of $MgSO_4$, 0·16 mg. of $FeCl_3$, 10 g. of mannitol, 30 mg. of adenine and 15 mg. of L-histidine hydrochloride. These concentrations of adenine and histidine are in the optimum range for growth.

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Bacteriophage. The T2r strain was prepared and assayed as described by Adams (1950) and Burton (1955).

iso*Citric dehydrogenase.* A partially purified preparation was kindly given by Dr H. L. Kornberg.

Nucleotides. Deoxyribonucleotides were obtained from the California Corporation for Biochemical Research (Los Angeles 63, Calif., U.S.A.) and other nucleotides from Sigma Chemical Co. (St Louis 18, Mo., U.S.A.).

Conditions of bacterial growth and bacteriophage infection. E. coli B-96 was grown on agar slants overnight at 37°. The growth medium, described above, was inoculated from this culture, aerated overnight at 37° and then added to 7 vol. of fresh growth medium. Aeration was continued until the concentration of cells was about 5×10^8/ml. (2–2½ hr.). The bacteria were centrifuged, washed at 4° with the original volume of buffered saline (Burton, 1955) and resuspended in growth medium at 37° in the absence of adenine. The bacteria were infected with an average of five infective T2r particles per cell and aeration was continued. Thereafter adenine was added as indicated.

The medium used to suspend the bacteria in the experiment of Table 3 had a low concentration of phosphate and contained (per l.) 10 g. of mannitol, 3 g. of NaCl, 27 mg. of KH_2PO_4, 10 mg. of $MgSO_4$, 0·16 mg. of $FeCl_3$ and 15 mg. of histidine hydrochloride and was buffered with 0·05M-triethanolamine (adjusted to pH 7·4 with HCl) and 0·01M-succinic acid (adjusted to pH 7·4 with NH_3).

Some difficulty was caused by unexplained variations in the growth rate of the bacteria. For the experiments which are described in detail, the cultures reached the required

concentration in 2–2½ hr. after inoculation from the overnight culture. Certain cultures for unknown reasons required more than 4 hr. Although they appeared to give normal growth of phage in the presence of adenine, they synthesized protein very slowly when infected with phage in the absence of adenine.

Preparation of cell-free extracts. Portions of uninfected and infected cultures were chilled and the bacteria were sedimented rapidly at 4°. The bacteria were suspended in 0·1 M-potassium phosphate buffer, pH 7·0, (2 ml./100 ml. of original culture) and disrupted for 3 min. in a Mullard-MSE ultrasonic disintegrator working at 90 kcyc./sec. and 60 w with a 1·9 cm. diam. stainless-steel parallel probe. The suspension was centrifuged at 3000 *g* for 15 min. The supernatant, designated 'cell-free extract', was stored at −20° until required.

Enzyme assays

Deoxycytidylate hydroxymethylase. This was assayed according to Flaks & Cohen (1959) with the inclusion of di- and tri-phosphopyridine nucleotide (each at 5 mM), 2·5 mM-DL-*iso*citrate and *iso*citrate dehydrogenase (0·1 mg./ml.). These additions slightly increased the activity, perhaps because they kept tetrahydropteroylglutamic acid in the reduced form. The reaction was stopped by addition of perchloric acid to a final concentration of 0·2 N; the acid-soluble fraction was neutralized with KOH. Potassium perchlorate was removed by centrifuging and portions of the supernatant were hydrolysed and plated as described by Flaks & Cohen (1959). The radioactivity was measured in a gas-flow proportional counter fitted with a thin Micromil window (Nuclear-Chicago Corp., Chicago 10, Ill., U.S.A.).

Deoxynucleotide kinases. For the assay of hydroxymethylcytidylate kinase (and also the deoxyribonucleotide polymerase), [^{14}C]hydroxymethylcytidylate was prepared enzymically according to Flaks & Cohen (1959) and purified by chromatography on Dowex-1 formate resin. The specific activity was calculated from the measured radioactivity and ultraviolet absorption, from the value of $13{\cdot}5 \times 10^3$ at 284 mμ and pH 1 for the molar extinction coefficient. The assay mixture contained 0·02 μmole of [^{14}C]hydroxymethylcytidylate (500 000 counts/min./μmole), 1·0 μmole of adenosine triphosphate (ATP), 4 μmoles of 2-amino-2-hydroxymethylpropane-1:3-diol brought to pH 7·8 with HCl, 1·0 μmole of $MgCl_2$ and enzyme containing 5–15 μg. of protein in a total volume of 0·1 ml. After 10 min. at 37°, the reaction was stopped by adding perchloric acid to a final concentration of 0·4 N. The acid soluble fraction was neutralized with KOH and the resulting potassium perchlorate was removed. The labelled nucleotides were separated on 2 cm. × 0·8 cm. diam. columns of Dowex-1 × 8 formate resin. First the monophosphate was eluted by 0·1 N-formic acid, then the diphosphate by 4 M-formic acid and finally the triphosphate by 2 N-HCl. The radioactivity in each fraction was determined. The ratio of the di- to triphosphate was found to vary with experimental conditions, but was always less than 0·5.

The other deoxyribonucleotide kinases were assayed by incubating 2·5 μmoles of thymidylate, deoxyguanylate or deoxyadenylate with a mixture of 100 μmoles of 2-amino-2-hydroxymethylpropane-1:3-diol adjusted to pH 7·8 with HCl, 10 μmoles of ATP, 10 μmoles of $MgCl_2$ and enzyme containing 0·3–1·0 mg. of protein in a total volume of 2·5 ml. Controls without deoxyribonucleotides were included. After 10 min. at 37° the reaction was stopped as described above. For the assay of the deoxyguanylate or thymidylate kinases the acid-soluble fraction was chromatographed on a 5·5 cm. × 0·8 cm. diam. column of Dowex-1 × 8 formate resin. All nucleotides, except deoxyguanosine triphosphate or thymidine triphosphate, were eluted with 4 M-formic acid plus 0·3 M-ammonium formate (about 70–80 ml.). The deoxyguanosine triphosphate or thymidine triphosphate was then eluted with M-ammonium formate (pH 5) and estimated spectrophotometrically. The deoxyribonucleoside diphosphates were usually not investigated because of the more tedious chromatography that is required for their separation. In one experiment they were isolated and found to represent about 10% of the triphosphates. For the assay of the deoxyadenylate kinase the deoxyadenosine triphosphate was determined after the ribonucleotides had been removed by periodate oxidation according to Lehman, Bessman, Simms & Kornberg (1958). The acid-soluble fraction was chromatographed on Dowex-1 × 8 formate columns, 5·5 cm. × 0·8 cm. diam. The ultraviolet-absorbing components except deoxyadenosine triphosphate were eluted by about 50 ml. of 4 M-formic acid plus 0·1 M-ammonium formate. Deoxyadenosine triphosphate was then eluted by 4 M-formic acid plus 0·3 M-ammonium formate.

Deoxyribonucleotide polymerase. This enzyme was assayed according to Lehman *et al.* (1958) by measuring the incorporation of [^{14}C]hydroxymethylcytidylate (500 000 counts/min./μmole) into deoxyribonucleic acid (DNA) in the presence of deoxyadenylate, deoxyguanylate, thymidylate and calf-thymus DNA which had been heated for 5 min. at 100° and pH 9. The incubation mixture contained 0·02 μmole of each deoxynucleotide, 3 μmoles each of ATP and $MgCl_2$, 15 μmoles of 2-amino-2-hydroxymethylpropane-1:3-diol adjusted to pH 7·8 with HCl, 0·1 mg. of DNA and enzyme containing 0·05–0·1 mg. of protein in a total volume of 0·3 ml. The incubation was for 30 min. at 37°. Under these conditions the deoxyribonucleoside monophosphates were converted into the triphosphates by the respective kinases and subsequently polymerized. The rates of phosphorylation are much higher than the rates of polymerization.

In all enzyme assays two levels of enzyme were used and the reactions were proportional to the enzyme concentration.

Isolation of ^{35}S-labelled protein

Portions (10 ml.) of the culture were precipitated with perchloric acid, washed with 5% trichloroacetic acid, extracted twice at 70° for 20 min. with 0·5 N-perchloric acid and washed twice with 0·5 N-perchloric acid. The residue was suspended in acetone, plated on filter paper and counted with an end-window Geiger–Müller tube.

Radioactive carbon in nucleic acid purines. Portions of the cultures (10 ml.) were added to 1 ml. of 55% (w/v) trichloroacetic acid. The precipitate was washed several times with 5% (w/v) trichloroacetic acid. Nucleic acids were extracted from it by 0·5 N-perchloric acid at 70°, the purines were obtained as their silver salts (cf. McDonald, 1954) and plated on filter paper for radioactivity measurement by an end-window Geiger–Müller tube. After counting, the silver salts were decomposed by 0·1 N-HCl and the amounts of purine determined spectrophotometrically (Loring, 1955). Paper chromatography in propan-2-ol–HCl (Wyatt, 1951) confirmed that adenine and guanine were the only ultraviolet-absorbing materials present. Under the

conditions used the counting rates of different samples from the same culture were approximately proportional to the amounts of purines.

Chemical analyses

DNA was estimated on 5 ml. portions of the cultures as described by Burton (1956) with deoxyribose as the standard. The apparent (i.e. purine-bound) deoxyribose was multiplied by 2 to give values for the total deoxyribose. Protein was determined according to Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Synthesis of purines

To test for the completeness of the genetic block in strain B-96, [1-^{14}C]glycine (5·4 mg. = 0·02 mc/l.) was added to normal and phage-infected bacteria in adenine-free medium. After 60 min., portions (10 ml.) of the cultures were taken and the radioactivities of the silver salts of the nucleic acid purines were measured. After correction for background radiation, the specific activities and standard deviations were $12{\cdot}3 \pm 7{\cdot}6$ counts/min./μmole in the uninfected culture and $16{\cdot}9 \pm 11{\cdot}8$ counts/min./μmole in the phage-infected culture. Analogous experiments with *E. coli* strain B indicated that activities of at least 1500 counts/min./μmole would have been found with this strain. There is therefore negligible incorporation of glycine into purines by strain B-96, although it is incorporated into protein at rates which are comparable with those in the parent strain B.

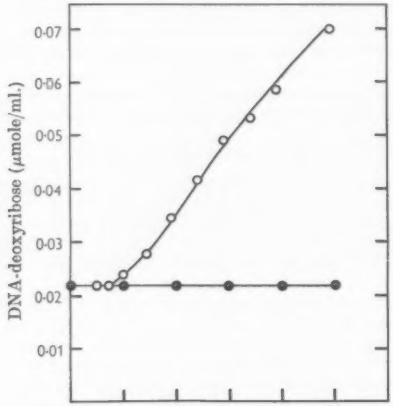

Fig. 1. Dependence of net DNA synthesis in infected cultures on the presence of adenine. At 0 min. two 50 ml. portions of washed cells were infected with phage T2. Adenine (30 mg./l.) was added to one portion. The cultures were aerated at 37°. ○, Adenine; ●, no added adenine.

Synthesis of deoxyribonucleic acid and proteins

As shown in Fig. 1, omission of adenine from the infected culture prevents an increase in the DNA content. This observation led us to investigate whether exogenous adenine is required solely for the net synthesis of DNA or whether it is also needed for the synthesis of ribonucleic acid (RNA) and protein. Fig. 2 shows that the absence of adenine decreases the incorporation of ^{35}S into the total proteins of the infected culture by only about 20 %. A similar result has been reported by Watanabe & Kiho (1958) in an experiment with another purine-requiring mutant of *E. coli*.

In the experiment in Fig. 3 the infected cultures were incubated in the absence of adenine for periods of 8 or 15 min. and then adenine was added together with chloramphenicol, which is expected to arrest any further protein synthesis. Under these conditions net synthesis of DNA occurred, indicating that the proteins needed for the synthesis of phage DNA were formed in the absence of adenine, although, as suggested by the experiment in Fig. 3 (*a*), the synthesis of the proteins may have been somewhat slower than in the presence of adenine.

Further evidence that these proteins are made in the absence of exogenous adenine is given by the

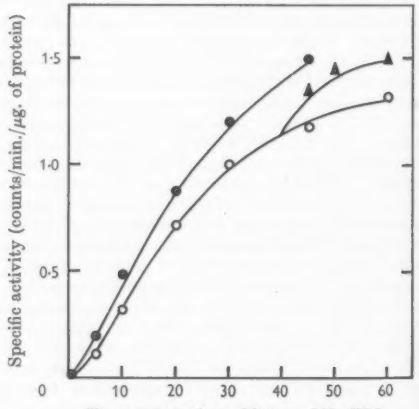

Fig. 2. Incorporation of ^{35}S into the total proteins of infected cells in the presence and absence of adenine. A washed culture was infected with an average of eight T2r particles/cell in a growth medium free of adenine and containing 10 mg. of $MgCl_2$ and 115 mg. of Na_2SO_4/l. instead of $MgSO_4$. Carrier-free $Na_2{}^{35}SO_4$ (0·18 mc/l.) was added 3 min. later and the culture was divided. One sample was incubated in the absence of adenine (○). After 40 min. a portion of the sample was removed, adenine (30 mg./l.) was added and the incubation was continued (▲). To another sample adenine (30 mg./l.) was added immediately after the $Na_2{}^{35}SO_4$ (●).

Table 1. *Base composition of deoxyribonucleic acid of* Escherichia coli B-96 *infected with* T2r *phage in the absence of adenine*

Analyses were made according to Hershey, Dixon & Chase (1953) on the DNA from portions (200 ml.) of culture both before and at various times after infection with phage in the absence of adenine. No explanation can be given for the low recoveries of adenine. Separate determinations on the same culture showed that the amount of DNA remained constant. The amounts of the bases are expressed as μmoles recovered/100 μmoles of total deoxyribose.

Time after infection (min.)	Hydroxymethyl-cytosine	Cytosine	Guanine	Thymine	Adenine	Base recovery (%)
0	0	24·7	23·6	26·0	17·7	92
15	1·3	22·7	25·0	26·7	18·3	94
30	3·7	15·7	22·3	27·7	18·8	88
45	6·7	11·0	21·0	29·7	20·0	88

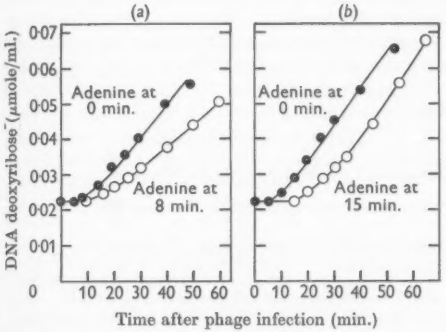

Fig. 3. Synthesis of DNA when adenine and chloramphenicol are added at different times after infection. In the same experiment as shown in Fig. 1, additional portions of the infected culture were aerated at 37°. Adenine (30 mg./l.) was added as indicated. Chloramphenicol (20 mg./l.) was added at 8 and 15 min. after infection in (*a*) and (*b*) respectively. To other portions of the culture not shown in the figure, chloramphenicol was added at 3 min. after infection and adenine at either 0 or 3 min. No change occurred in the DNA content.

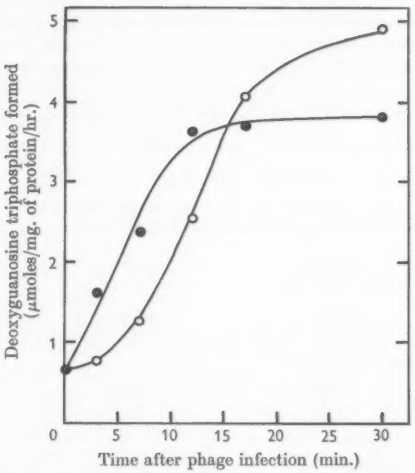

Fig. 4. Level of deoxyguanylate kinase in *Escherichia coli* B-96 infected with T2r bacteriophage in the presence (●) and absence (○) of adenine. Assay was as described in the Methods section.

conversion of bacterial DNA into phage DNA. This is indicated by the results in Table 1, which show a loss of cytosine and the appearance of hydroxymethylcytosine in the DNA of infected cells.

More direct evidence that some of the necessary enzymes are formed in the absence of exogenous adenine is presented in Fig. 4 and Table 2. Whether adenine is present or not the enzyme levels reach a similar maximal level, although the rate of enzyme synthesis is somewhat slower in the absence of adenine.

For undefined reasons some cultures of *E. coli* B-96 grew more slowly than normal cultures. If they were infected in the presence of adenine when they had reached a concentration of about 5×10^8 cells/ml. they synthesized DNA and the necessary enzymes at about the same rate as described above. However, in the absence of adenine the enzymes began to be made only after a much longer period of infection. Similarly, if cultures grown at the normal rate were kept in an adenine-free medium for 5 min. before infection the synthesis of protein was markedly reduced in the cultures devoid of exogenous adenine.

Synthesis of a structural protein of the phage was examined by measuring the serum-blocking power essentially according to DeMars (1955). Whether adenine was present or not, the levels detected were of the same order of magnitude and appreciable serum-blocking power was first observed between 20 and 30 min. after infection. The results are not presented in detail because they were variable and do not permit strictly quantitative comparisons.

Ribonucleic acid synthesis

A number of the 'early enzymes' are not found in the host bacteria, and, like the structural proteins of the phage, they are probably new proteins synthesized after the infection (Flaks & Cohen, 1957, 1959; Kornberg, Zimmerman, Kornberg & Josse, 1959; Koerner, Smith & Buchanan, 1959; Somerville, Ebisuzaki & Greenberg, 1959; Bessman, 1959; Bessman & Van Bibber, 1959). If RNA plays a role in the synthesis of proteins (review Loftfield, 1958) it is expected that new RNA would be synthesized in the infected cultures grown in the absence of adenine from precursors contained in the bacteria. Volkin & Astrachan (1956) and Astrachan & Volkin (1959) demonstrated that, although phage infection arrests net RNA synthesis, there is RNA turnover which appears to involve the synthesis of a different kind of RNA, as shown by an altered distribution of ^{32}P among the four nucleotides. This effect of the phage is most marked in the presence of chloramphenicol. As is shown in Table 3, it occurs in cultures which are devoid of exogenous adenine, although these cultures incorporated less ^{32}P into RNA (about one-seventh of that incorporated in the presence of adenine). Other experiments in the absence of chloramphenicol indicated that infection in the absence of adenine caused a similar change in the distribution of ^{32}P among the nucleotides obtained from the RNA, and there was also a reduction to about one-third of the incorporation in the presence of adenine.

Watanabe & Kiho (1958) have also reported that the changed pattern of incorporation occurs in the absence of adenine. It suggests that exogenous adenine is not required for the synthesis of a new type of RNA molecule. Although the lower incorporation of ^{32}P in the absence of adenine might suggest a slower turnover of RNA, it could also be due to a decreased entry of ^{32}P into the nucleotide precursors of RNA.

Table 2. *Level of enzymes involved in the synthesis of phage deoxyribonucleic acid*

The activities of all the enzymes shown except deoxyadenylate kinase were measured on the same five cell-free extracts by methods described in the text.

		Enzyme activities (μmoles/mg. of protein/hr.)				
			8 min. after infection		15 min. after infection	
Enzyme	Basis of assay	Uninfected	No adenine	Adenine	No adenine	Adenine
Deoxycytidylate hydroxymethylase	Incorporation of H·^{14}CHO	0	0·20	0·20	0·30	0·30
Deoxyribonucleotide kinases	Formation of					
Hydroxymethyl-cytidylate	Di- and tri-phosphate	0	0·78	1·50	2·58	2·58
Thymidylate	Triphosphate	0·47	0·91	1·89	3·39	3·05
Deoxyguanylate	Triphosphate	0·65	1·28	2·42	4·09	3·66
Deoxyadenylate	Triphosphate	3*	—	—	—	—
Deoxyribonucleotide polymerase	Incorporation of [^{14}C]-hydroxymethylcytidylate	Nil†	0·001	0·004	0·007	0·006

* The activity was also approximately the same at 5 and 12 min. after infection.
† [^{14}C]Hydroxymethylcytidylate is not phosphorylated and is therefore not incorporated into DNA.

Table 3. *Incorporation of ^{32}P-labelled orthophosphate into ribonucleic acid*

Chloramphenicol (4·5 mg.) was added to a 150 ml. portion of a washed culture in low-phosphate medium devoid of adenine. After 3 min. a tracer dose of carrier-free $Na_2H^{32}PO_4$ (0·066 mc) was added and 10 min. later the culture was chilled and centrifuged. Another 150 ml. portion of the washed culture was infected with T2r phage. After 3 min., 4·5 mg. of chloramphenicol was added; 6 min. after infection 0·066 mc of $Na_2H^{32}PO_4$ was added and 10 min. later the cells were chilled and centrifuged. The RNA nucleotides were isolated as described by Hecht & Potter (1956).

	Specific activities			
	Counts/min./μmole		Relative to guanylic acid	
Nucleotide	Uninfected	Infected	Uninfected	Infected
Guanylic acid	2820	3310	(1·0)	(1·0)
Cytidylic acid	3620	3910	1·3	1·2
Adenylic acid	3590	5980	1·3	1·8
Uridylic acid	3750	6570	1·3	2·0

DISCUSSION

The results indicate that, after phage infection, the enzymes required for the synthesis of phage DNA (Flaks & Cohen, 1957, 1959; Koerner *et al.* 1959; Kornberg *et al.* 1959; Somerville *et al.* 1959; Bessman, 1959) are made whether exogenous purines are supplied or not. The extent of phage DNA synthesis is, however, controlled by the supply of preformed purines. In the absence of an exogenous purine source limited phage DNA synthesis can occur. Under these conditions some of the components of the bacterial DNA, which is degraded after infection, are apparently utilized for the synthesis of the new type of DNA, but a further supply of purines derived from other bacterial components does not seem to be available for DNA synthesis, since there is no net change in the DNA concentration.

The results obtained are not inconsistent with current concepts of the role of RNA in protein synthesis (review Loftfield, 1958) since the new RNA appears to be formed even under conditions where the purine supply is limited and where there is no net synthesis of nucleic acids.

SUMMARY

1. When a purine-requiring mutant (B-96) of *Escherichia coli* is infected with T2r phage, net synthesis of deoxyribonucleic acid requires a supply of exogenous purine. Direct and indirect evidence is presented that exogenous purines are not required for the synthesis of either the structural proteins of the phage or the enzymes necessary for the synthesis of phage deoxyribonucleic acid.

2. Even when exogenous adenine is not supplied, the nucleic acids of the bacterial cell undergo conversions which characteristically follow infection with phage T2. Cytosine of the deoxyribonucleic acid is replaced by 5-hydroxymethylcytosine and ^{32}P-labelled inorganic phosphate is incorporated into ribonucleic acid with an altered distribution among the nucleotides obtained by alkaline hydrolysis.

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REFERENCES

Adams, M. H. (1950). *Meth. med. Res.* **2**, 1.
Astrachan, L. & Volkin, E. (1959). *Biochim. biophys. Acta*, **32**, 449.
Bessman, M. J. (1959). *J. biol. Chem.* **234**, 2735.
Bessman, M. J. & Van Bibber, M. J. (1959). *Biochem. Biophys. Res. Commun.* **1**, 101.
Burton, K. (1955). *Biochem. J.* **61**, 473.
Burton, K. (1956). *Biochem. J.* **62**, 315.
DeMars, R. I. (1955). *Virology*, **1**, 83.
Flaks, J. G. & Cohen, S. S. (1957). *Biochim. biophys. Acta*, **25**, 667.
Flaks, J. G. & Cohen, S. S. (1959). *J. biol. Chem.* **234**, 1501.
Gots, J. S. (1950). *Arch. Biochem.* **29**, 222.
Gots, J. S. (1953). *Nature, Lond.*, **172**, 256.
Hecht, L. I. & Potter, V. R. (1956). *Cancer Res.* **16**, 999.
Hershey, A. D., Dixon, J. & Chase, M. (1953). *J. gen. Physiol.* **36**, 777.
Koerner, J. F., Smith, M. S. & Buchanan, J. M. (1959). *J. Amer. chem. Soc.* **81**, 2594.
Kornberg, A., Zimmerman, S. B., Kornberg, S. R. & Josse, J. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 772.
Lehman, I. R., Bessman, M. J., Simms, E. S. & Kornberg, A. (1958). *J. biol. Chem.* **233**, 163.
Loftfield, R. B. (1958). *Progress in Biophysics and Biochemistry*. London: Pergamon Press Ltd.
Loring, H. S. (1955). In *The Nucleic Acids*, vol. 1. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
McDonald, I. W. (1954). *Biochem. J.* **57**, 566.
Somerville, R., Ebisuzaki, K. & Greenberg, G. R. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 1240.
Volkin, E. & Astrachan, L. (1956). *Virology*, **2**, 149.
Watanabe, I. & Kiho, Y. (1958). In *Proceedings of the International Symposium on Enzyme Chemistry, Tokyo and Kyoto*. London: Pergamon Press Ltd.
Wyatt, G. R. (1951). *Biochem. J.* **48**, 581.

The Reaction of Carbohydrases with Phenolic Glucosides

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The biochemistry and physiology of phenolic glycosides in plants has been reviewed by Pridham (1960*a*). The biosynthesis of a phenolic diglucoside was first recorded by Miller (1941), who fed *o*-chlorophenol to *Gladiolus* corms and isolated the corresponding *o*-chlorophenyl β-gentiobioside. The enzymic formation of *p*-hydroxyphenyl β-gentiobioside from arbutin (*p*-hydroxyphenyl β-D-glucopyranoside) with a β-glucosidase preparation from aspen cambium (*Populus grandidentata*) has also been reported (Pridham, 1957, 1960*b*). With wheat-germ enzymes, however, the formation of this diglucoside from arbutin required uridine diphosphoglucose as a cofactor (Cardini & Yamaha, 1958, 1960). These wheat-germ extracts showed no β-glucosidase activity.

A study of the reaction of arbutin with enzyme preparations from almond (*Prunus amygdalus*) and broad bean (*Vicia faba* var. Johnson's Longpod) seeds suggests that β-glucosidase can catalyse the formation of a phenolic gentiobioside. When salicin (*o*-hydroxymethylphenyl β-D-glucopyranoside) was used as a substrate, *o*-hydroxybenzyl β-glucoside was produced, thus substantiating the early observations of Rabaté (1935), who investigated a similar reaction of salicin with an enzyme from willow (*Salix purpurea*) leaves.

MATERIALS AND METHODS

Ultraviolet-absorption spectra were measured in a Unicam SP. 500 spectrophotometer (1 cm. cell). Infrared measurements were made with a Unicam SP. 100 double-beam spectrophotometer. Control incubations were in all instances carried out with boiled enzyme preparations and all enzyme reactions were carried out in the presence of toluene.

Paper chromatography. Phenolic compounds and sugars were examined on Whatman no. 1 and no. 3 papers, respectively, by the descending technique. The solvent systems were: *A*, butan-1-ol–pyridine–water (6:4:3, by vol.); *B*, butan-1-ol–ethanol–water (40:11:19, by vol.) and *C*, ethyl acetate–acetic acid–water (9:2:2, by vol.). Diazotized *p*-nitroaniline–Na_2CO_3 (Swain, 1953) spray reagent was used to detect phenols and *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950), to detect the reducing sugars.

Paper electrophoresis. Whatman no. 3 paper and 0·1 M-sodium borate buffer (pH 10·0) was used as described by Pridham (1959). Mobilities are expressed as M_{SA} values, i.e. relative to the mobility of salicylic acid.

p-*Hydroxyphenyl β-cellobioside.* This was synthesized by the method of Stánek & Kocourek (1953).

Enzyme preparations. Almond emulsin was prepared by the method described by Sumner & Somers (1947). Before use the enzyme solution was dialysed against running tap water (40 hr.) and then distilled water (5 hr.). Bean tissue was macerated with 0·07 M-sodium phosphate (pH 7·0) buffer containing L-cysteine (0·5%, w/v) (about 1 g. of tissue/5 ml. of buffer) at 5°. The resulting extract was then centrifuged at 3000 rev./min. and the supernatant liquor dialysed against distilled water for 24 hr. at 5°. Enzyme preparations were made from (*a*) whole seeds (with testas removed) which had been soaked in water for 18 hr. at room temperature, (*b*) dormant embryos, (*c*) embryos from seeds treated as in (*a*), (*d*) embryos from germinating seeds which had been kept between layers of moist cotton wool for 6 days at room temperature, (*e*) cotyledons from seeds treated as in (*a*). Preparation (*a*) was fractionated by the addition of solid $(NH_4)_2SO_4$ to give 50% saturation, the mixture was then put into a separating funnel and the precipitated protein allowed to rise to the surface. The clear solution was run off and the suspended protein dialysed against distilled water (24 hr., 5°) and freeze-dried. Preparations (*c*) and (*e*) were both dialysed successively against three changes of distilled water (4 days), 0·05 M-sodium acetate, pH 5 (24 hr.) and 0·07 M-sodium phosphate, pH 8·5 (24 hr.). The resulting solutions were then adjusted to pH 6·5 with acetic acid.

Each enzyme preparation was incubated with 2·0% (w/v) solutions of salicin and arbutin for 18 hr. at 27° and the reaction mixtures were examined on paper chromatograms for sugars and phenols. The incubations with arbutin were repeated in the presence of D-glucono-1→4-lactone (0·7%, w/v) for the emulsin and the enzyme preparations (*a*) and (*b*).

Compound (I). Arbutin (3 g.) was incubated with emulsin solution (50 ml.) for 5 days at 37°; the incubation mixture was deproteinized by the method of Sevag, Lackman & Smollens (1938) and then extracted with ether to remove quinol. The aqueous solution was then passed through a small charcoal–Celite column (Whistler & Durso, 1950) which was washed with water to remove glucose. Aqueous ethanol (50%, v/v) was used to elute the glycosides from the column and the eluate was then concentrated under reduced pressure to a thin syrup. A phenolic compound (I) (30 mg.) was obtained from the latter by chromatographic fractionation on Whatman no. 3MM paper (solvent *B*). Complete hydrolysis of (I) was effected

by heating with N-H_2SO_4 (100°; 2 hr.) and also by treating with emulsin (27°, 18 hr.). Heating with N-HCl (100°, 10 min.) resulted in a partial hydrolysis of (I). Compound (I) (5 mg.) in water (1 ml.) was treated with bromine water (0·2 M, 0·5 ml.) in the presence of Amberlite IR-4B (OH^- form) resin to neutralize any HBr formed. The solution was aerated to remove excess of Br_2 and then filtered and concentrated under reduced pressure to a syrup.

The optimum pH values for the formation of compound (I) with emulsin and with preparation (*a*) were estimated by incubating these enzymes (1 ml.) at 27° for 18 hr. with arbutin (5·0%, w/v) and either 0·1 M-sodium citrate or 0·07 M-sodium phosphate buffers (2 ml., pH range 3·0–8·0) and observing the rates at which compound (I) was formed, by paper chromatographic examination. Attempts to synthesize arbutin or compound (I), or both, were made by incubating 1 ml. of emulsin, preparation (*a*) or β-glucosidase (0·1%, w/v, aqueous solution; Sigma Chemical Co.) with quinol (20 mg.) and salicin, cellobiose, maltose or α-D-glucose 1-phosphate (100 mg. of each) in the presence of 0·05 M-sodium acetate buffer (1 ml., pH 5·6) for 18 hr. at 27°.

Compound (II). Salicin (2 g.) was incubated with emulsin (66 ml.) for 7 days at 37°. The reaction mixture was then deproteinized and extracted with ether, as with compound (I), and the remaining solution concentrated and fractionated on Whatman no. 3MM paper (solvent *B*). Hydrolysis with N-H_2SO_4 and emulsin was carried out as with compound (I).

RESULTS

Paper-chromatographic examination of the products obtained by incubating arbutin with emulsin revealed the presence of glucose, quinol and a slower-moving component (I) (Table 1), which gave the same purple colour as arbutin with the diazonium spray reagent and also reacted with *p*-anisidine periodate (Bragg & Hough, 1958); small amounts of other compounds were also detected. In aqueous solution the spectrum of (I) resembled that of arbutin with an absorption maximum at 283 mμ (cf. Charlampowiczówna & Marchlewski, 1930). After addition of bromine water, this peak was replaced by two other maxima at 246 and 268 mμ which are characteristic of *p*-benzoquinone (cf. Braude, 1945). The solution after bromine treatment contained a compound which gave a brown colour with *p*-anisidine hydrochloride and co-chromatographed with authentic gentiobiose. (Gentiobiose was clearly distinguished from all other glucopyranosyl disaccharides by solvent *C*.) This evidence suggested that compound (I) was *p*-hydroxyphenyl gentiobioside. Complete acid hydrolysis of the gentiobioside yielded glucose and quinol and a partial hydrolysis gave a disaccharide which was chromatographically identical with gentiobiose. Treatment of the gentiobioside with emulsin produced glucose, quinol, and arbutin, thus suggesting the presence of β-links in the molecule; this was confirmed by infrared measurements on compound (I), which showed a characteristic absorption band at 891 $cm.^{-1}$ (Barker, Bourne, Stacey & Whiffen, 1954). The behaviour of the β-gentiobioside (I) differed from that of authentic *p*-hydroxyphenyl β-cellobioside on paper chromatography and electrophoresis (Table 1).

Incubation of arbutin with enzyme preparations from broad-bean tissues also produced a compound identical with *p*-hydroxyphenyl β-gentiobioside and the pH optimum for the formation of this compound was between 5 and 6 for both the bean [preparation (*a*)] and emulsin. All enzyme preparations which were tested produced the β-gentiobioside when incubated with arbutin. They also hydrolysed the latter compound, and salicin, to glucose and the corresponding phenolic aglycone. This reversible β-glucosidase activity was inhibited by D-glucono-1 → 4-lactone with emulsin and enzyme preparations (*b*) and (*a*). The protein fraction (*a*) obtained by precipitation with 50% ammonium sulphate saturation retained its ability to synthesize *p*-hydroxyphenyl β-gentiobioside as did preparations (*c*) and (*e*). Unsuccessful attempts were made to prepare arbutin and *p*-hydroxyphenyl β-gentiobioside by incubating emulsin [commercial and Sumner & Somers (1947) preparations] and bean enzymes with quinol and various glucose derivatives. *p*-Hydroxyphenyl β-gentiobioside was not produced when bean seeds or shoots were allowed to take up solutions of quinol (0·25%) or arbutin (0·5%) under the conditions of Pridham (1958) and Pridham & Saltmarsh (1960).

When the almond or bean enzyme preparations were incubated with salicin, the following were formed: glucose, saligenin (*o*-hydroxybenzyl alcohol) and a phenolic compound (II). Saligenin and (II) gave the same red colour with the

Table 1. *R_F and M_{SA} values of phenolic glucosides*

Glucoside	R_F in solvents			M_{SA} in 0·1 M-sodium borate buffer (pH 10)
	A	*B*	*C*	
Arbutin	0·75	0·40	0·51	—
p-Hydroxyphenyl β-gentiobioside (I)	0·55	0·07	0·17	0·28
p-Hydroxyphenyl β-cellobioside	0·61	0·10	0·24	0·22
Salicin	—	0·56	0·48	0
o-Hydroxybenzyl β-glucoside (II)	—	0·59	0·48	0·15

diazonium reagent and both had similar u.v.-absorption spectra with maxima at 275 and 274 mμ, respectively (cf. Ramart-Lucas & Rabaté, 1933). In the presence of 0·002N-NaOH the absorption maximum of (II) shifted to 294 mμ. Salicin had approximately the same R_F value as (II) (Table 1) but did not react with the diazonium reagent. However, these two compounds were clearly separated by paper electrophoresis (Table 1). The absorption maximum of salicin at 268 mμ (cf. Charlampowiczówna & Marchlewski, 1930) was not affected by the addition of alkali. Glucose and saligenin only were detected in the acid and emulsin hydrolysates of (II).

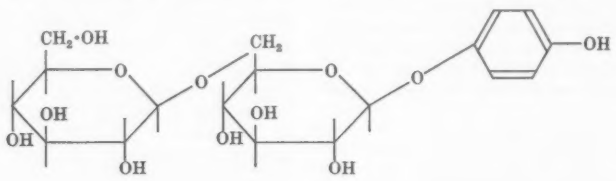
DISCUSSION

The results obtained show that compound (I) is *p*-hydroxyphenyl β-gentiobioside, which was formed directly from arbutin. That the transfer of β-D-glucosyl units by β-glucosidase is involved with both the almond and bean preparations is suggested by the fact that β-glucopyranosides were hydrolysed by all of the enzyme solutions tested and that this activity and the ability to synthesize the gentiobioside was inhibited by D-glucono-1 → 4-lactone, a competitive inhibitor of β-glucosidase (Conchie & Levvy, 1955). In addition, the pH optima for β-glucosidase activity and the formation of the gentiobioside are similar and the synthesis of a 1 → 6 β-link by D-glucopyranosyl transfer involving β-glucosidase might be expected from the observations of Peat, Whelan & Hinson (1952) and Barker, Bourne, Hewitt & Stacey (1955).

The fact that extensively dialysed enzyme solutions and a protein fraction obtained by ammonium sulphate precipitation all synthesized the phenolic gentiobioside makes it unlikely that uridine diphosphoglucose participates in the reaction although the possibility that this nucleotide is present in a bound form cannot be ignored (cf. Buell, 1952).

The biosynthesis of phenolic diglucosides *in vivo* is more likely to involve uridine diphosphoglucose than a simple glucose donor, e.g. a disaccharide, in conjunction with β-glucosidase. It also seems probable that monoglucosylation of a phenolic hydroxyl group *in vivo* and *in vitro* requires the presence of a 'high-energy' glucose donor and that simple transglucosylation reactions are of little importance for this process (Pridham & Saltmarsh, 1960).

Compound (II) is an isomer of salicin with the β-glucopyranosyl group attached to the primary hydroxyl group of the saligenin nucleus. The u.v.-absorption measurements and paper electrophoresis indicated the presence of a free phenolic hydroxyl group. In this instance β-glucosidase would appear to have catalysed the transfer of a glucosyl radical from the phenolic to the primary hydroxyl group in the side chain with the formation of *o*-hydroxybenzyl β-glucopyranoside. This glycoside is also formed when glucose is incubated with saligenin in the presence of emulsin (Bourquelot & Hérissey, 1913), by the action of a willow-leaf extract on salicin (Rabaté, 1935) and by allowing broad-bean shoots and seeds to take up solutions of saligenin (Pridham, 1958; Pridham & Saltmarsh, 1960).



(I)

The possibility of glycoside synthesis should be borne in mind when carrying out kinetic studies on glycosidases, with phenolic or alcoholic glycosides as substrates, otherwise anomalous results may be obtained.

SUMMARY

1. The incubation of almond emulsin, or enzyme preparations from broad bean, with arbutin produces *p*-hydroxyphenyl β-gentiobioside.

2. A transglucosylation reaction catalysed by β-glucosidase is involved with arbutin molecules serving as both donors and acceptors of D-glucopyranosyl residues.

3. Salicin is partially converted into *o*-hydroxybenzyl β-glucopyranoside by the almond and broad-bean preparations.

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REFERENCES

Barker, S. A., Bourne, E. J., Hewitt, G. C. & Stacey, M. (1955). *J. chem. Soc.* p. 3734.

Barker, S. A., Bourne, E. J., Stacey, M. & Whiffen, D. H. (1954). *J. chem. Soc.* p. 171.

Bourquelot, E. & Hérissey, H. (1913). *C.R. Acad. Sci., Paris*, **156**, 1790.
Bragg, P. D. & Hough, L. (1958). *J. chem. Soc.* p. 4050.
Braude, E. A. (1945). *J. chem. Soc.* p. 492.
Buell, M. V. (1952). *Fed. Proc.* **11**, 192.
Cardini, C. E. & Yamaha, T. (1958). *Nature, Lond.*, **182**, 1446.
Cardini, C. E. & Yamaha, T. (1960). *Arch. Biochem. Biophys.* **86**, 133.
Charlampowiczówna, B. & Marchlewski, L. (1930). *C.R. Soc. Polon. Phys.* p. 376.
Conchie, J. & Levvy, G. A. (1955). *Biochem. J.* **61**, xvi.
Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.
Miller, L. P. (1941). *Contr. Boyce Thompson Inst.* **12**, 163.
Peat, S., Whelan, W. J. & Hinson, K. A. (1952). *Nature, Lond.*, **170**, 1056.
Pridham, J. B. (1957). *Analyt. Chem.* **29**, 1167.
Pridham, J. B. (1958). *Nature, Lond.*, **182**, 795.
Pridham, J. B. (1959). *J. Chromat.* **2**, 605.
Pridham, J. B. (1960*a*). In *Phenolics in Plants in Health and Disease*. Ed. by Pridham, J. B. London: Pergamon Press Ltd. (in the Press).
Pridham, J. B. (1960*b*). *Biochem. J.* **76**, 13.
Pridham, J. B. & Saltmarsh, M. J. (1960). *Biochem. J.* **74**, 42P.
Rabaté, J. (1935). *Bull. Soc. Chim. biol., Paris*, **17**, 572.
Ramart-Lucas, P. & Rabaté, J. (1933). *C.R. Acad. Sci., Paris*, **196**, 1493.
Sevag, M. G., Lackman, D. B. & Smollens, J. (1938). *J. biol. Chem.* **124**, 425.
Stănek, J. & Kocourek, J. (1953). *Chem. Listy*, **47**, 697.
Sumner, J. B. & Somers, G. F. (1947). *Chemistry and Methods of Enzymes*, p. 96. New York: Academic Press Inc.
Swain, T. (1953). *Biochem. J.* **53**, 200.
Whistler, R. L. & Durso, D. F. (1950). *J. Amer. chem. Soc.* **72**, 677.

Biochem. J. (1960) **77**, 567

The Acid-Soluble Nucleotides of Codling (*Gadus callarias*) Muscle

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The literature contains few references to the nucleotides of fish muscle. Tarr (1950) and Fujimaki & Kojô (1953) investigated a number of Pacific Ocean species by the fractionation of extracts with barium, and Partmann (1954) measured the adenosine 5′-triphosphate contents of tench and pike muscles. Steinbach (1949), Davison & Richards (1954) and Partmann (1954, 1957, 1958) have examined the apyrase activities occurring in fish muscle under different conditions. In preliminary communications (Jones & Murray, 1957; Murray & Jones, 1958; Jones, 1958) we described separations of the major constituents of the nucleotide fraction in codling muscle during exercise and after death. These studies were carried out by formate elution from anion-exchange resin columns (Hurlbert, Schmitz, Brumm & Potter, 1954). More recently, Saito & Arai (1958*a*, *b*) and Saito, Arai & Yajima (1959) have reported separations of the major nucleotides from carp and rainbow trout, using a chloride-elution technique.

This paper describes the identification of both the major and minor nucleotides of codling muscle in detail. Additionally, it extends our earlier quantitative data.

EXPERIMENTAL

Materials

Reference compounds for chromatography. Nucleotides were purchased from Sigma Chemical Co., St Louis, Mo. Guanosine and cytidine were purchased from Roche Products Ltd., Welwyn Garden City, Herts., adenosine, inosine, adenine, hypoxanthine, cytosine and guanine from L. Light and Co. Ltd., Colnbrook, Bucks., and uracil from British Drug Houses Ltd., Poole, Dorset.

Codling. These were caught off Aberdeen by trawl and kept in circulating sea water until landed. The fish were physically exhausted and some were killed in this condition. Others were kept ashore in aerated sea water for 8 weeks on a diet of squid. After this time, the fish were in a good nutritional condition and were well-rested when killed.

Methods

Killing. The fish, which were 18–24 in. long, were stunned by a blow on the head and dropped immediately into liquid oxygen. Preliminary experiments had ascertained that this procedure gave concentrations of labile phosphorus (measured as described below) comparable with those in the muscle of fish made comatose by immersion in sea water containing 1% (w/v) of urethane before killing. The concentrations of adenosine 5′-triphosphate (ATP) measured were significantly higher than those obtained by dropping the fish into liquid oxygen without stunning.

Extraction. The frozen skin was levered away with a thin chisel and the exposed blocks of muscle were readily separated from the skeleton by blows from a hammer. Pieces (60 g.) from the part of the fish immediately anterior to the dorsal fin and above the lateral line were ground in the frozen condition and homogenized into 120 ml. of chilled 0·6N-perchloric acid. The homogenates were filtered at 0° and samples equivalent to 50 g. of tissue were adjusted to pH 6·5 with a 30% (v/v) soln. of potassium hydroxide. After 30 min. at 0°, potassium perchlorate was removed by centrifuging and the extract was stored at −30° to await analysis.

Ion-exchange chromatography of extracts. The thawed extract was run through a column (17·5 cm. × 1·8 cm.) of 200–400 mesh Dowex-1, ×8, (formate) and the column was then washed with water until the effluent was free from material absorbing at 260 mμ. The procedures for the initial preparation of the column and the subsequent 'stepwise-gradient' elution were based essentially on those of Hurlbert *et al.* (1954). The nucleotides were eluted from the column by a solution from a mixing vessel containing a constant 500 ml. of liquid. Into the initial 500 ml. of water in this vessel were mixed successively 2N-formic acid (470 ml.), 4N-formic acid (960 ml.), 4N-formic acid containing 0·2M-ammonium formate (880 ml.), 4N-formic acid containing 0·4M-ammonium formate (850 ml.) and 4N-formic acid containing 0·8M-ammonium formate (500 ml.). This system of elution gave a more gradual rise in the concentration of formate ion during the early stages of separation than the original procedure. The congestion of the early peaks was thereby lessened.

Spectrophotometric examination of fractions. A Unicam SP. 500 instrument was used. Quantitative evaluations of the bases in the fractions were made by reference to the molar extinction coefficients proposed by Bock, Ling, Morrell & Lipton (1956) and Dorough & Seaton (1954). Corrections were made for any absorption of light by the eluent. Suitable 'blanks' were used in the examination of preparations from fractions.

Cleavage of the pyrimidine ring for spectrophotometric measurement. The preparations were brominated and treated subsequently with dilute alkali (Cohn, 1956).

Estimation of total phosphorus. The fractions were digested by the procedure of LePage (1949) and the phosphorus content of the digest was measured by that of Fiske & Subbarow (1925).

Estimation of labile phosphorus. The phosphorus liberated by heating the preparation in N-sulphuric acid for 15 min. at 100° was determined as above.

Estimation of ribose. The method of Mejbaum (1939) was used. Purine derivatives were treated directly with the orcinol reagent. Pyrimidine compounds were brominated and treated with alkali as above to obtain a quantitative recovery of ribose after the cleavage of the ring.

Absorption of nucleotides on charcoal and their elution. The procedure of Baddiley, Buchanan, Carss, Mathias & Sanderson (1956) was followed up to the stage of freeze-drying. In our hands a faint turbidity, due to a fine suspension of carbon, always accompanied the elution of the nucleotides from Norit A although the charcoal had been pre-washed repeatedly. However, the addition of 4 vol. of chloroform to the aq. ammonia–water–ethanol eluate, followed by an immediate centrifuging, resulted in the separation of the interfering carbon at the interface of the two phases which formed. The upper, aqueous phase contained the nucleotides. It was freeze-dried immediately over phosphorus pentoxide or fractionated with barium.

Removal of formate and ammonium formate from fractions. Formic acid was removed *in vacuo* from peaks 1 and 2 (see below). Except where otherwise stated, formic acid and ammonium formate were removed from later peaks by charcoal treatment as above.

Fractionation of nucleotides with barium. Fractions which had been freed from formate by adsorption on the charcoal and elution as above were fractionated with barium acetate (LePage, 1949).

Estimation of diphosphopyridine nucleotide. A cyanide addition reaction (Ciotti & Kaplan, 1957) was used.

Hydrolysis of nucleotides. Purine bases were liberated with N-hydrochloric acid at 100° for 1 hr. (Markham & Smith, 1950) or with N-sulphuric acid at 100° for 1 hr. (Vischer & Chargaff, 1948; Loring, Fairley, Bortner & Seagran, 1952). Pyrimidine nucleoside di- and tri-phosphates were hydrolysed to monophosphates by the same hydrochloric acid treatment. Free pyrimidine bases were freed by heating the preparations with concentrated formic acid at 175° for 2 hr. (Vischer & Chargaff, 1948). Formic acid and hydrochloric acid were removed from the hydrolysates *in vacuo* over potassium hydroxide. Sulphuric acid was removed as the barium salt.

Phosphate ester linkages in the nucleotides were split by alkaline hydrolysis with 5% (w/v) ammonia solution at 145° for 3–5 hr. (Levene & Jacobs, 1910). The ammonia was removed *in vacuo* over phosphorus pentoxide.

Paper chromatography. The chromatograms were run on Whatman no. 1 or no. 4 paper, previously washed with ethylenediaminetetra-acetic acid (EDTA) solution (Eggleston & Hems, 1952), except for sugar chromatography, when unwashed papers were used. Solvents used for the separation of nucleotides in fractions were (1) *iso*butyric-acid–ammonia–EDTA (Krebs & Hems, 1953), (2) propan-1-ol–ammonia (Webster, 1953), (3) pyridine–water (Burton & San Pietro, 1954) and (4) phenol–water–8-hydroxyquinoline (Hummel & Lindberg, 1949). Nucleosides, purines and pyrimidines were separated with (5) butan-1-ol–formic acid and (6) butan-1-ol–ammonia (Markham & Smith, 1949) or with (7) propan-2-ol–hydrochloric acid (Wyatt, 1951). Sugars were separated with (8) phenol–ammonia (no hydrocyanic acid) and (9) butan-1-ol–acetic acid (Partridge, 1948). Glucuronic acid and glucurone were chromatographed with (10) ethanol–ammonium acetate (Storey & Dutton, 1955). In the Results section, solvents are referred to by the numbers in parentheses above.

The free purine and pyrimidine bases and compounds containing them were detected on the chromatograms as dark spots against a fluorescent back-ground under a Hanovia Chromatolite lamp. The position of spots absorbing ultraviolet light weakly was established by ultraviolet photography (Markham & Smith, 1949) with the same lamp. Bases were also detected by spraying the papers with ethanolic phenol and counter-spraying with a commercial hypochlorite solution (Harris & Parsons, 1956). Phosphorylated compounds were detected with molybdate reagent (Hanes & Isherwood, 1949), sugars with aniline hydrogen phthalate (Partridge, 1949*a*) and glucuronic acid and glucurone with aniline oxalate (Partridge, 1949 *b*). Compounds containing ribose but no pyrimidine residue were detected by an adaptation of the Mejbaum (1939)

procedure for reaction with orcinol in solution. The developed chromatograms were dried and sprayed with a solution of 15% (w/v) hydrochloric acid containing 0·35% (w/v) of orcinol and 0·035% (w/v) of ferric chloride. They were heated in a stream of air at 80° until purple spots appeared against the yellowish background. The limit of detection was 4–5 μg. of ribose. Pyridinium compounds were detected by their fluorescence in ultraviolet light when papers were sprayed with N-potassium cyanide.

RESULTS

Separation of nucleotides and examination of peaks

Fig. 1 illustrates a typical separation of components from the muscle of rested codling. The compositions of the five major and nine lesser peaks were examined. The latter were frozen, stored at −30° and peaks from several separations were combined to obtain sufficient material for investigation.

With most of the peaks referred to below, position in the pattern of elution (cf. Hurlbert *et al.* 1954) was a valuable identification point.

Peak 1. This contained only diphosphopyridine nucleotide (DPN). The absorption spectra showed $\lambda_{max.}$ 260 mμ in 2N-hydrochloric acid, $\lambda_{max.}$ 260 mμ and 325 mμ in cyanide. Chromatograms in solvents 1 and 4 showed the presence of a single compound which absorbed ultraviolet light, reacted with molybdate reagent, fluoresced in ultraviolet light when sprayed with potassium cyanide and ran in the position of marker DPN.

Peak 2. Adenosine 5′-monophosphate (AMP) and contaminants from the adjacent peak 3 were present. The absorption spectra showed: $\lambda_{max.}$ 258 mμ, $\lambda_{min.}$ 230 mμ in 2N-hydrochloric acid; $\lambda_{max.}$ 260 mμ, $\lambda_{min.}$ 227 mμ in N-sodium hydroxide, indicating adenine base. Chromatography in solvents 1 and 2 and the estimation of ultraviolet-light-absorbing material by quantitative spot-elution (Markham & Smith, 1949) showed that 96% ran as a discrete spot, in the position of AMP, and reacted with molybdate reagent. On overloaded chromatograms the remaining material appeared to be the same as the constituents of peak 3. Chromatography of the products of hydrolysis by N-hydrochloric acid in solvents 5, 6, and 7 showed only adenine under ultraviolet light. Analysis gave adenine:ribose:phosphate as 1:0·98:1·09 equivalent proportions (expected proportions, 1:1:1).

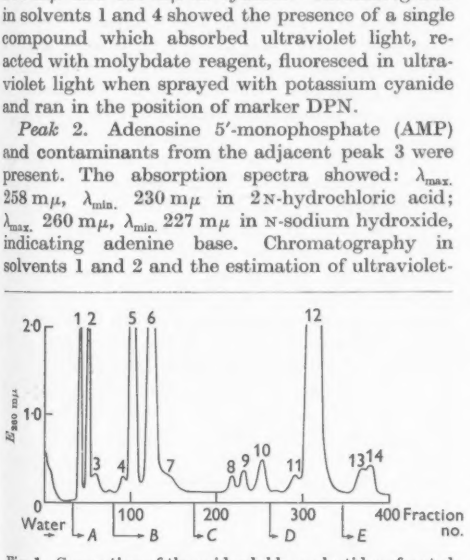

Fig. 1. Separation of the acid-soluble nucleotides of rested codling muscle. Extract, equivalent to 50 g. of muscle, was separated on Dowex-1 by stepwise-gradient elution (see Methods section). Fraction size was 10 ml. Eluting solvents were: *A*, 2N-formic acid; *B*, 4N-formic acid; *C*, 4N-formic acid + 0·2M-ammonium formate; *D*, 4N-formic acid + 0·4M-ammonium formate; *E*, 4N-formic acid + 0·8M-ammonium formate.

Peak 3. This contained taurine, contaminating AMP from peak 2, unidentified phosphorylated material which fluoresced in ultraviolet light and traces of two unidentified ninhydrin-reacting substances. The absorption spectrum showed a poorly defined $\lambda_{max.}$ at 260 mμ in 2N-hydrochloric acid. Chromatograms in solvent 1 showed AMP and a blue-fluorescing streak covering much of their length in ultraviolet light. Molybdate reagent gave a weak reaction to this streaking material, to orthophosphate and AMP. Ninhydrin in butan-1-ol (Jones, 1955) gave a spot in the region of AMP.

Fractions equivalent to 40 g. of muscle were shaken in 20 ml. of water with 5 g. of 100–200 mesh sulphonated polystyrene resin, of 4% nominal divinyl bonding, in the hydrogen form (Permutit Co. Ltd., Gunnersbury Avenue, London, W. 4) for 15 min. at 15°. The ninhydrin-reacting fraction separated into two components. That not held by the resin was estimated by quantitative spot-elution (Jones, 1955) to account for some 70% of the reactivity with ninhydrin: two-dimensional chromatography in phenol–ammonia and collidine–lutidine (Dent, 1948) showed it to comprise taurine and a trace of AMP. The other component was eluted from the resin with 0·25N-ammonium solution. Two-dimensional chromatography, as described above, demonstrated the presence of AMP, confirmed by chromatography in solvent 2, and traces of two other unidentified compounds, which reacted with ninhydrin but not molybdate and did not absorb ultraviolet light.

Peak 4. This contained a mixture of guanosine 5′-monophosphate with lesser amounts of triphosphopyridine nucleotide, an unidentified compound, which fluoresced in ultraviolet light, and inosine 5′-monophosphate from peak 5. The constituents of the mixture were investigated by absorption spectra and chromatography. The spectra showed: $\lambda_{max.}$ 257 mμ, $\lambda_{min.}$ 230 mμ and an inflexion at 275–280 mμ in 2N-hydrochloric acid; $\lambda_{max.}$ 257 mμ, $\lambda_{min.}$ 230 mμ with higher relative absorption at 280 mμ in N-sodium hydroxide. In N-potassium cyanide, a secondary peak of $\lambda_{max.}$ 325 mμ ($E_{325\ m\mu} = 8\%\ E_{257\ m\mu}$) was ob-

tained. These spectra indicated the presence of guanosine nucleotide with a lesser quantity of a pyridinium compound, probably triphosphopyridine nucleotide. Chromatograms in solvent 1 showed a strong spot which reacted with molybdate reagent and absorbed ultraviolet light in the position of guanosine 5′-monophosphate and triphosphopyridine nucleotide (identical R_F values). Another spot, which ran more slowly than orthophosphate, reacted with molybdate and fluoresced with a blue colour in the presence and absence of cyanide. On hydrolysis with formic acid and chromatography in solvents 6 and 7, adenine, guanine, and a weaker indication of hypoxanthine, poorly separated from the latter, were found. The presence of hypoxanthine was confirmed when the papers were sprayed with phenol and hypochlorite. Chromatography of the hydrolysate in solvents 1, 6 and 7 failed to detect the original blue-fluorescing compound.

Peak 5. This peak contained 99 % inosine 5′-monophosphate and 1 % pyrimidine nucleotides, probably cytidine 5′-diphosphate and uridine 5′-monophosphate. After fractionation with barium and ethanol and the removal of barium as the sulphate, the absorption spectra showed $\lambda_{max.}$ 252 mμ, $\lambda_{min.}$ 223 mμ in 2N-hydrochloric acid and $\lambda_{max.}$ 255 mμ, $\lambda_{min.}$ 227 mμ in N-sodium hydroxide, indicating an inosine nucleotide, slightly contaminated with material which had a higher $\lambda_{max.}$. Chromatograms of the barium-fractionated material in solvent 1 showed only inosine 5′-monophosphate. The first two fractions of the peak from other separations were chromatographed as above, without prior barium fractionation. The chromatograms, heavily overloaded with respect to inosine 5′-monophosphate, had a weak indication of material absorbing ultraviolet light and reacting with molybdate in the position of cytidine 5′-diphosphate. This material had $\lambda_{max.}$ 280 mμ in 2N-hydrochloric acid and $\lambda_{max.}$ 270 mμ at pH 13, confirming the presence of cytidine nucleotide. Similar chromatograms of the late fractions of the peak gave an indication of uridine 5′-monophosphate but there was considerable interference from the main component.

On hydrolysis in N-hydrochloric acid and chromatography in solvents 6 and 7, only hypoxanthine, was detectable in ultraviolet light. On overloaded chromatograms in solvent 7 a suggestion of reaction with molybdate reagent was noticed in the position of cytidine 5′-monophosphate. Any reaction in the position of uridine monophosphate was obscured by the very large orthophosphate spot. Bromination of a sample from the peak, to cleave pyrimidines without prior charcoal treatment, resulted in an increase of 1·4 % of the original ribose value (mean of five determinations).

Peak 6. This contained adenosine diphosphate (ADP) which was identified together with AMP, a breakdown product, by chromatography in solvents 1, 2 and 3 and by absorption spectra. These showed $\lambda_{max.}$ 257 mμ, $\lambda_{min.}$ 230 mμ in 2N-hydrochloric acid; $\lambda_{max.}$ 260 mμ, $\lambda_{min.}$ 227 mμ in N-sodium hydroxide. On hydrolysis with N-hydrochloric acid, adenine was the only ultraviolet-light-absorbing material found with solvents 5 and 7. Analysis of the peak gave adenine:ribose:phosphate, 1:1·03:1·95 in equivalent proportions (expected proportions 1:1:2).

Peak 7. This contained either a single cytidine nucleotide which suffered some breakdown during charcoal purification or two different cytidine nucleotides, neither of which has been identified. Together with ADP and its breakdown product AMP, contaminants from peak 6, chromatograms in solvent 1 showed two weaker ultraviolet-light-absorbing spots which ran respectively anterior to and contiguous to the ADP position. These were not identified. The absorption spectra of both of the eluted spots were similar. They had poorly-defined λ_{max} in the region of 276–280 mμ in 2N-hydrochloric acid and 270–275 mμ in N-sodium hydroxide. Bromination destroyed this absorption. These spectra were characteristic of cytidine nucleotide. Bromination also increased the recovery of ribose from the peak by 68 % of the value obtained without treatment. Chromatography in solvent 7 showed that a hydrolysate in formic acid contained adenine and either cytosine or cytidine, which had identical R_F values.

Peak 8. This was identified as uridine 5′-diphosphate acetylglucosamine on the following evidence. The absorption spectra showed $\lambda_{max.}$ 261 mμ, $\lambda_{min.}$ 230 mμ in 2N-hydrochloric acid; $\lambda_{min.}$ 240 mμ in N-sodium hydroxide; bromination destroyed this absorption. Uridine 5′-monophosphate and traces of the 5′-diphosphate were found on chromatograms in solvent 1: when formate was removed from the peak by freeze-drying and sublimation (Hurlbert *et al.* 1954), the diphosphate gave the stronger spot. The formate-free material gave a weak reaction for *N*-acetylglucosamine (Morgan & Elson, 1934). Analysis gave 0·08 equiv. of ribose increasing to 0·93 equiv. on bromination: 0·88 equiv. of labile phosphorus: 1·79 equiv. of total phosphorus: 1 equiv. proportion of uracil (expected 1:1:2:1). The sugar moiety of uridine 5′-diphosphate acetylglucosamine is known to be labile under the conditions of formate removal (Hurlbert & Potter, 1954) and this accounted for the identification of uridine 5′-diphosphate and the monophosphate on paper chromatograms.

Peak 9. This was identified as a mixture of guanosine 5′-diphosphate and uridine 5′-diphos-

phate glucose. As evidence of this the absorption spectra showed $\lambda_{max.}$ 260 mμ, $\lambda_{min.}$ 230 mμ, inflexion 270–285 mμ in 2N-hydrochloric acid; $\lambda_{max.}$ 260 mμ, $\lambda_{min.}$ 234 mμ, inflexion 265–272 mμ in N-sodium hydroxide; $E_{inflex.}/E_{max.}$ was higher in alkali. Guanosine 5′-diphosphate and/or uridine 5′-diphosphate (which had the same R_F) were found, with traces of the 5′-monophosphates, on chromatograms in solvent 1. After hydrolysis in formic acid and chromatography in solvents 6 and 7, spots absorbing ultraviolet light were found in the position of guanine and uracil; hydrolysis in ammonia yielded guanosine and uridine. After hydrolysis with 0·01N-hydrochloric acid the preparation contained glucose and ribose as shown on chromatograms in solvents 8 and 9, sprayed with aniline hydrogen phthalate; glucose was also present in unhydrolysed preparations. From considerations similar to those for peak 8 this would derive from the labile uridine 5′-diphosphate glucose.

Peak 10. This appeared to contain a fraction analogous to the 'AdX' isolated from rat liver by Hurlbert *et al.* (1954). The composition of this material has not been established but it is thought to contain derivatives of adenosine substituted in two positions by phosphate. The absorption spectra of the peak showed $\lambda_{max.}$ 257 mμ, $\lambda_{min.}$ 230 mμ in 2N-hydrochloric acid; $\lambda_{max.}$ 259 mμ, $\lambda_{min.}$ 227 mμ in N-sodium hydroxide, indicating adenosine nucleotide. After hydrolysis with hydrochloric acid and subsequent fractionation with barium to remove remaining nucleotide (LePage, 1949) the preparation was shown by chromatography in solvents 6 and 7 to contain only adenine. The nucleotide material, chromatographed in solvent 1 yielded two spots which absorbed ultraviolet light, reacted to molybdate reagent and ran slightly more slowly than ADP and ATP. Analysis gave adenine:ribose:phosphate in 1:0·91:2·68 equivalent proportions. Bromination did not increase the recovery of ribose. Only 38% of the phosphate was liberated by 2N-sulphuric acid at 100° in 15 min.

Peak 11. On chromatographic evidence, this contained cytidine 5′-triphosphate, uridine 5′-diphosphate and traces of uridine 5′-diphosphate glucuronic acid. With solvent 1, material absorbing ultraviolet light and reacting to molybdate reagent, streaked throughout the length of the chromatograms. Orcinol-sprayed chromatograms showed only ATP, ADP and AMP deriving from the contiguous peak 12, indicating that much of the material absorbing ultraviolet light was not purine nucleotide. After hydrolysis with hydrochloric acid, the acid-free residue equivalent to 50 g. of tissue was dissolved in 5 ml. of water. This was shaken at pH 6·5 with 200 mg. of 200–400 mesh, Dowex-1 (chloride) resin for 15 min. to remove phosphorylated compounds. Chromatograms of the supernatant solution in solvents 6 and 7 showed only adenine. The resin was eluted with 2N-hydrochloric acid (10 ml.). Chromatograms of the acid-free eluate in solvent 1 showed spots absorbing ultraviolet light and reacting to molybdate in the positions of cytidine 5′-monophosphate and uridine 5′-monophosphate. The presence of cytidine 5′-monophosphate was confirmed by the absorption spectra of the eluted spot: $\lambda_{max.}$ 280 mμ, $\lambda_{min.}$ 242 mμ in 2N-hydrochloric acid; $\lambda_{max.}$ 272 mμ, $\lambda_{min.}$ 253 mμ in N-sodium hydroxide. That of uridine 5′-monophosphate was confirmed similarly: $\lambda_{max.}$ 261 mμ, $\lambda_{min.}$ 230 mμ in 2N-hydrochloric acid; $\lambda_{min.}$ 242 mμ in sodium hydroxide. After hydrolysis in N-sulphuric acid, chromatography in solvent 10 yielded a spot reacting with aniline oxalate in the position of ribose and traces of colour in the positions of glucuronic acid and glucurone. From the considerations above, glucuronic acid and its derivative would be present originally as uridine 5′-diphosphate glucuronic acid.

Peak 12. This contained ATP and 2% of another compound containing adenine base. The absorption spectra were characteristic of adenosine nucleotides: $\lambda_{max.}$ 257 mμ, $\lambda_{min.}$ 230 mμ in 2N-hydrochloric acid; $\lambda_{max.}$ 259 mμ, $\lambda_{min.}$ 227 mμ in N-sodium hydroxide. In addition to ATP and lesser quantities of AMP and ADP, present as breakdown products, chromatograms in solvent 1 showed a small ultraviolet-light-absorbing spot running a little faster than ATP and not completely separated from it. When eluted, this material had an adenine-type absorption curve, $\lambda_{max.}$ 258 mμ, $\lambda_{min.}$ 230 mμ in 0·2N-hydrochloric acid. The absorption due to this material was 2–4% of the total absorption at 260 mμ. Chromatograms in solvents 2 and 3 confirmed the presence of ATP, AMP and ADP. On hydrolysis with N-hydrochloric acid adenine was the only material absorbing ultraviolet light found with solvents 6 and 7. Analysis of the whole peak gave adenine:ribose:phosphate 1:0·94:2·96 in equivalent proportions (expected for ATP 1:1:3).

Peak 13. This contained guanosine 5′-triphosphate together with uridine 5′-triphosphate from peak 14, from which it was poorly separated. Chromatograms in solvents 1 and 2 showed a number of spots which absorbed ultraviolet light and reacted with molybdate reagent. With the possible exception of a large spot running in the position of guanosine 5′-triphosphate and uridine 5′-triphosphate, none of these separated well enough for identification. However, spraying with orcinol confirmed the presence of guanosine 5′-triphosphate and showed lesser, distorted spots of

guanosine 5′-monophosphate and guanosine 5′-diphosphate. The material absorbing ultraviolet light on the remainder of the chromatograms gave no orcinol reaction. A hydrolysate in N-hydrochloric acid yielded a large spot absorbing ultraviolet light in the position of guanine on chromatograms in solvent 7 and a lesser spot, which reacted with molybdate reagent, in the position of uridine 5′-monophosphate. The absorption spectrum of the large spot, eluted into 2N-hydrochloric acid, showed $\lambda_{max.}$ 257 mμ, $\lambda_{min.}$ 228 mμ, inflexion at 270–280 mμ. In N-sodium hydroxide $\lambda_{max.}$ was 257 mμ, $\lambda_{min.}$ 230 mμ; the inflexion shifted to a slightly lower wavelength and $E_{270\ m\mu}$ approached $E_{257\ m\mu}$, indicating guanine.

Peak 14. This contained primarily uridine 5′-triphosphate, with guanosine 5′-triphosphate from peak 13. Chromatograms in solvent 1 were similar to those of peak 13 except with orcinol spray, when the pattern of spots was considerably weaker. The major spot absorbing ultraviolet light, running in the position of uridine 5′-triphosphate or guanosine 5′-triphosphate, gave on elution $\lambda_{max.}$ 260 mμ, $\lambda_{min.}$ 230 mμ in 2N-hydrochloric acid; $\lambda_{max.}$ 258 mμ, $\lambda_{min.}$ 234 mμ in N-sodium hydroxide. A slight inflexion in the region of 270 mμ was very much less pronounced than would be expected of a pure guanosine nucleotide. Bromination destroyed the absorption. These spectra indicated uridine nucleotide as the major constituent. A hydrolysate in hydrochloric acid was fractionated with Dowex-1 (chloride) as for peak 11. The non-phosphorylated, ultraviolet light-absorbing material was shown by chromatography in solvent no. 7 to be guanine. Chromatography in solvents no. 1 and 7 showed that the nucleotide fraction eluted from the resin contained only uridine 5′-monophosphate. The absorption of this material at 260 mμ was estimated by spot elution to be 82% of that of the original preparation.

Table 1. *Acid-soluble-nucleotide content of rested and exhausted codling muscle*

Nucleotides were separated as in Fig. 1. DPN and triphosphopyridine nucleotide were estimated by the cyanide-addition reaction and the measurement of absorption at 325 mμ on the peaks containing them. AMP was estimated from its absorption at 260 mμ, assuming that it had separated symmetrically from the contiguous peak 3 about the fraction of minimum absorption. Inosine 5′-monophosphate was determined similarly after deducting an arbitrary 1% (see Results, peak 5) for contamination by pyrimidine nucleotides. ADP and ATP were also estimated similarly, 2% being deducted from the triphosphate value and none from that of the diphosphate. Uridine 5′-triphosphate and guanosine 5′-phosphate were estimated on the mixed peaks 13 and 14 by differential spectrophotometry at 261 and 281 mμ at pH 1. Blank values of the formate eluent were deducted from all peaks. Values are mean determinations on six fish.

Nucleotide	Content in muscle (μmoles/g.)	
	Rested	Exhausted
Diphosphopyridine nucleotide	0·107	0·338
Triphosphopyridine nucleotide	0·0008	0·0005
Adenosine 5′-monophosphate	0·69	0·57
Adenosine 5′-diphosphate	0·576	0·426
Adenosine 5′-triphosphate	5·34	0·260
Inosine 5′-monophosphate	1·26	5·86
Guanosine 5′-triphosphate	0·0025	0
Uridine 5′-triphosphate	0·0036	0

Estimation of the nucleotides in codling muscle

Having established the composition of the major peaks, it was possible to estimate their components with reasonable accuracy. Table 1 illustrates the differences in composition between muscle from rested and exercised, exhausted fish. Over 80% of the ATP of the rested muscle was dephosphorylated and deaminated to inosine 5′-monophosphate during the struggle to exhaustion and the 5′-triphosphates of uridine, guanosine and cytidine disappeared. Conversely the level of DPN increased.

DISCUSSION

The general picture of the nucleotide separations from codling muscle reported in this paper is broadly similar to those of other mammalian and fish tissues investigated by this procedure (e.g. Hurlbert *et al.* 1954; Manson, 1956; Tsuyuki, Chang & Idler, 1958; Keir & Davidson, 1958). As might be expected from their role in contraction, the main characteristic of the codling muscle separations was the high concentration of ATP or its breakdown product inosine 5′-monophosphate.

The concentration of ATP in the rested muscle (5·34 μmoles/g.) was slightly lower than that (6·8 μmoles/g.) reported for rabbit muscle (Bendall & Davey, 1957) from data obtained by the monochloroacetate elution of nucleotides from anion-exchange resin. The value agrees well, however, with that (5·76 μmoles/g.) obtained for carp muscle (Saito & Arai, 1958*b*) by chloride elution. More recently Saito *et al.* (1959) reported a slightly lower concentration (3·53 μmoles/g.) in the dorsal muscle of freshly killed rainbow trout. Severe exercise lowered the concentration in codling muscle to 0·260 μmole/g., a value similar to those obtained in many instances by the early workers, fractionating fish muscle extracts with barium. As Tarr (1958) has pointed out, much of the earlier data on fish muscle (e.g. Tarr, 1950; Partmann, 1954; Fujimaki & Kojô, 1953) were obtained from material which had either struggled before death or which had been dead for some time before extraction for

analysis. These very low concentrations in exercised fish are interesting in relation to current theories of the development of *rigor mortis*. They are considerably lower than the critical plasticizing concentration in mammalian muscle at room temperature (Bate-Smith & Bendall, 1956) and there may well be significant species differences in adenine nucleotide metabolism.

The ADP content of muscle from both rested (0·576μmole/g.) and exhausted (0·426μmole/g.) codling was lower than that recorded (0·94μmole/g.) for carp (Saito & Arai, 1958*b*), rainbow trout (0·93–1·04μmoles/g.; Saito *et al.* 1959) and rabbit muscle (0·9μmole/g.; Bendall & Davey, 1957). Possibly, these lower concentrations may reflect the greater resolving power of the formate elution system, but the uniformity of the ADP concentration in the muscles of other species (cf. Bendall & Davey, 1957) estimated by different methods leads us to suppose that the low value in codling muscle is an individual species characteristic. Bendall & Davey (1957) have presented evidence that ADP rather than the triphosphate is stoicheiometrically related to the actomyosin content of the muscle filaments.

AMP was a minor constituent of the muscle relative to its inosine analogue. The values (0·69 and 0·57μmole/g. in rested and exhausted muscle respectively) were of the same order as that (0·42μmole/g.) present in the dorsal muscle of rainbow trout (Saito *et al.* 1959) but considerably higher than that (0·04μmole/g.) in carp muscle (Saito & Arai, 1958*b*).

Inosine 5′-monophosphate was a major constituent of the flesh of both rested (1·26μmoles/g.) and exhausted (5·86μmoles/g.) codling. Saito *et al.* (1959) reported 3·36μmoles/g. in rainbow-trout muscle, but Saito & Arai (1958*b*) found none in the flesh of freshly killed carp. These variations reflect the difficulty in obtaining some species in a completely rested condition.

The combined concentrations of adenine nucleotides (excepting DPN) and inosine 5′-monophosphate were slightly higher in the muscle of rested codling (7·24μmoles/g.) than in that of the exhausted fish (6·60μmoles/g.). Other work in this Laboratory has shown that this difference is accounted for by higher concentrations of inosine in the exhausted fish. The total nucleotide concentrations are in reasonable agreement with that (6·74μmoles/g.) of carp (Saito & Arai, 1958*b*) and trout (6·47–7·80μmoles/g.) (Saito *et al.* 1959). They are lower, however, than those determined by these authors for the dorsal muscle of rainbow trout (8·35μmoles/g.) and mackerel (9·30–10·7μmoles/g.). In general, it appears that the total nucleotide content of mammalian muscle [for example rabbit, in which Bendall & Davey (1957) found 11·7μmoles/g.] is higher than that in fish species.

Earlier workers have not investigated the minor acid-soluble constituents of fish muscle. The difference between the DPN contents of the rested and exhausted muscle was not significant when the pyridine nucleotide composition of a number of unfractionated extracts was measured in a separate study in this Laboratory by the direct determination of absorption at 325 mμ in the presence of cyanide. The concentrations in the muscle of both rested and exhausted fish were very variable. Nevertheless, by comparison with separations of the nucleotide components of rabbit muscle (Bendall & Davey, 1957; Newton & Perry, 1960), the DPN fraction of codling muscle was considerably larger. In contrast, the triphosphopyridine nucleotide content of codling muscle was very small. The significance of the other adenine-containing nucleotide ('ADX', peak 10) present in the muscle is not known. It has been observed in other tissues (cf. Hurlbert *et al.* 1954).

The unidentified ultraviolet-light-absorbing material of peak 3 has been found only in the muscle of rested codling. That of the exhausted fish we have examined has shown no trace of a peak in this position.

A small group of uridine 5′-diphosphate sugar-transferring enzymes has been identified in codling muscle. Uridine 5′-diphosphate glucose (peak 9) has been implicated in glycogen synthesis by pigeon-muscle microsomes (Robbins, Traut & Lipmann, 1959) and rat muscle (Leloir, Olavarría, Goldemberg & Carminatti, 1959). Villar-Palasi & Larner (1958) and Hauk & Brown (1959) have also described uridine 5′-diphosphate coenzyme-linked pathways of glycogen metabolism in muscle. The occurrence of uridine 5′-diphosphate acetylglucosamine and uridine 5′-diphosphate glucuronic acid in our preparation of codling muscle probably reflects the presence of myocommata in addition to the myotomes proper in the dissected tissue and also of muscle which had occupied positions immediately adjacent to bone. The presence of the coenzymes of systems transferring *N*-acetylglucosamine and glucuronic acid in such positions is not unlikely in such sites (cf. Storey & Dutton, 1955; Leloir, 1953).

Uridine 5′-triphosphate and guanosine 5′-triphosphate, which occurred as minor constituents of rested muscle, were not detectable in exercised material. Inosine 5′-triphosphate has been found in neither rested nor exhausted material in the present study. Saito & Arai (1958*a*, *b*) and Saito *et al.* (1959) did not report its presence in other fish species. Newton & Perry (1960), however, found the nucleotide in the muscle of freshly killed rabbits, although Bendall & Davey (1957) had

failed to do so. Neither we nor the Japanese school have detected inosine 5′-diphosphate in fish muscle. This could explain the absence of the 5′-triphosphate, which is formed in pigeon-breast muscle by the action of a nucleoside diphosphate phosphokinase (Krebs & Hems, 1953). The lower nucleotides of guanosine and uridine have been found in codling muscle, mixed in peaks with other constituents so that their accurate evaluation has not been possible. Of the cytidine nucleotides, the 5′-triphosphate and 5′-diphosphate have been identified in rested muscle. The 5′-monophosphate, which would be eluted in the region of DPN, has not been found in fresh muscle but other work in this laboratory has identified it in the spoiling material. The composition of a fourth, cytidine-containing material (peak 7) has not been elucidated.

From this study and those of Saito and his collaborators it appears that the metabolism of adenine and hypoxanthine nucleotides in fish muscle may differ in some respects from that of higher vertebrates. Further investigations are being carried out to establish any such differences more fully.

SUMMARY

1. The acid-soluble nucleotides in the skeletal muscle of rested and exercised codling have been separated by a formate elution system on anion-exchange resin, identified and estimated.

2. The 5′-mono-, 5′-di- and 5′-tri-phosphates of adenosine, guanosine and uridine were found. Of the cytidine nucleotides the 5′-monophosphate and of the inosine nucleotides the 5′-diphosphate and triphosphate were absent. Uridine 5′-diphosphate sugar-transferring coenzymes and an unidentified cytidine nucleotide were also detected.

3. Over 80% of the major component of the nucleotide fraction of rested muscle, adenosine 5′-triphosphate, was converted into inosine 5′-monophosphate during severe exercise. The absence of inosine 5′-di- and 5′-tri-phosphate may indicate a difference in nucleotide metabolism from that in higher vertebrates.

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REFERENCES

Baddiley, J., Buchanan, J. G., Carss, B., Mathias, A. P. & Sanderson, A. R. (1956). *Biochem. J.* **64**, 599.

Bate-Smith, E. C. & Bendall, J. R. (1956). *Brit. med. Bull.* **12**, 230.

Bendall, J. R. & Davey, C. L. (1957). *Biochim. biophys. Acta*, **26**, 93.

Bock, R. M., Ling, N-S., Morrell, S. A. & Lipton, S. H. (1956). *Arch. Biochem. Biophys.* **62**, 253.

Burton, R. M. & San Pietro, A. (1954). *Arch. Biochem. Biophys.* **48**, 184.

Ciotti, M. M. & Kaplan, N. O. (1957). In *Methods in Enzymology*, vol. 3, p. 890. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Cohn, W. E. (1956). *Biochem. J.* **64**, 28P.

Davison, J. A. & Richards, A. G. (1954). *Arch. Biochem. Biophys.* **48**, 484.

Dent, C. E. (1948). *Biochem. J.* **43**, 169.

Dorough, G. D. & Seaton, D. L. (1954). *J. Amer. chem. Soc.* **76**, 2873.

Eggleston, L. V. & Hems, R. (1952). *Biochem. J.* **52**, 156.

Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.

Fujimaki, M. & Kojô, K. (1953). *Bull. Jap. Soc. sci. Fish.* **19**, 499.

Hanes, G. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.

Harris, G. & Parsons, R. (1956). *Chem. & Ind.* p. 1312.

Hauk, R. & Brown, D. H. (1959). *Biochim. biophys. Acta*, **33**, 556.

Hummel, J. P. & Lindberg, O. (1949). *J. biol. Chem.* **180**, 1.

Hurlbert, R. B. & Potter, V. R. (1954). *J. biol. Chem.* **209**, 1.

Hurlbert, R. B., Schmitz, H., Brumm, A. F. & Potter, V. R. (1954). *J. biol. Chem.* **209**, 23.

Jones, N. R. (1955). *J. Sci. Fd Agric.* **6**, 3.

Jones, N. R. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, no. 7–17, p. 85.

Jones, N. R. & Murray, J. (1957). *Biochem. J.* **66**, 5P.

Keir, H. M. & Davidson, J. N. (1958). *Arch. Biochem. Biophys.* **77**, 1.

Krebs, H. A. & Hems, R. (1953). *Biochim. biophys. Acta*, **12**, 172.

Leloir, L. F. (1953). *Advanc. Enzymol.* **14**, 197.

Leloir, L. F., Olavarría, J. M., Goldemberg, S. H. & Carminatti, H. (1959). *Arch. Biochem. Biophys.* **81**, 508.

LePage, G. A. (1949). In *Manometric Techniques and Tissue Metabolism*, 2nd ed. p. 185. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.

Levene, P. A. & Jacobs, W. A. (1910). *Ber. dtsch. chem. Ges.* **43**, 3150.

Loring, H. S., Fairley, J. L., Bortner, H. W. & Seagran, H. L. (1952). *J. biol. Chem.* **197**, 809.

Manson, M. (1956). *Biochim. biophys. Acta*, **19**, 398.

Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.

Markham, R. & Smith, J. D. (1950). *Biochem. J.* **46**, 513.

Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **285**, 117.

Morgan, W. T. J. & Elson, L. A. (1934). *Biochem. J.* **28**, 988.

Murray, J. & Jones, N. R. (1958). *Biochem. J.* **68**, 9P.

Newton, A. A. & Perry, S. V. (1960). *Biochem. J.* **74**, 127.

Partmann, W. (1954). *Symposium on Cured and Frozen Fish Technology*, no. 17. Göteborg: Swedish Institute of Food Preservation Research.

Partmann, W. (1957). *Food Res.* **22**, 51.

Partmann, W. (1958). *Naturwissenschaften*, **45**, 213.

Partridge, S. M. (1948). *Biochem. J.* **42**, 238.

Partridge, S. M. (1949*a*). *Nature, Lond.*, **164**, 443.

Partridge, S. M. (1949*b*). *Symp. biochem. Soc.* **3**, 52.

Robbins, P. W., Traut, R. R. & Lipmann, F. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 6.

Saito, T. & Arai, K. (1958*a*). *Arch. Biochem. Biophys.* **73**, 315.

Saito, T. & Arai, K. (1958*b*). *Bull. Jap. Soc. sci. Fish.* **23**, 579.
Saito, T., Arai, K. & Yajima, T. (1959). *Nature, Lond.*, **184**, 1415.
Steinbach, H. B. (1949). *J. cell. comp. Physiol.* **33**, 123.
Storey, I. D. E. & Dutton, G. J. (1955). *Biochem. J.* **59**, 279.
Tarr, H. L. A. (1950). *J. Fish. Res. Bd Can.* **7**, 608.
Tarr, H. L. A. (1958). *Annu. Rev. Biochem.* **27**, 223.
Tsuyuki, H., Chang, V. M. & Idler, D. R. (1958). *Canad. J. Biochem. Physiol.* **36**, 465.
Villar-Palasi, C. & Larner, J. (1958). *Biochim. biophys. Acta*, **30**, 449.
Vischer, E. & Chargaff, E. (1948). *J. biol. Chem.* **176**, 715.
Webster, H. L. (1953). *Nature, Lond.*, **172**, 453.
Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.

Biochem. J. (1960) **77**, 575

Carnosine–Anserine Synthetase of Muscle

4. PARTIAL PURIFICATION OF THE ENZYME AND FURTHER STUDIES OF β-ALANYL PEPTIDE SYNTHESIS*

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Previous papers of this series (Winnick & Winnick, 1958, 1959*a*, 1960) have dealt with the general properties of carnosine–anserine synthetase, an enzyme which promotes the condensation of β-alanine with either L-histidine or 1-methylhistidine at neutral pH, in the presence of magnesium ions and adenosine triphosphate. The procedure for preparing carnosine–anserine synthetase involved ethanol fractionation of an extract of chick pectoral muscle. This method was employed by Kalyankar & Meister (1959) in their enzymic synthesis of a series of β-alanyl and γ-aminobutyryl peptides of histidine and various histidine derivatives.

Since a number of questions could not be answered satisfactorily with the crude enzyme, and in view of the current dearth of information relative to the biosynthesis of natural peptides, it was of interest to undertake the further purification and characterization of carnosine–anserine synthetase. Difficulties were encountered owing to the instability of the enzyme, and the study of the mechanism of peptide bond synthesis has been further complicated by the persistence of interfering enzymes in the partially purified preparation. However, certain properties of the latter have been established.

EXPERIMENTAL

Materials

DL-[1-^{14}C]Histidine, DL-1-methyl[1-^{14}C]histidine, DL-1-ethyl[1-^{14}C]histidine, and L-[^{3}H]carnosine (tritiated) were preparations previously described (Winnick & Winnick, 1958, 1959*a*). [^{32}P]Ortho- and [^{32}P]pyro-phosphate were products of The Radiochemical Centre, Amersham, Bucks. Nucleotides were obtained from Sigma Chemical Co., St Louis, Mo., and crystalline ribonuclease from Worthington Biochemical Corp., Freehold, N.J. DL-1-Methylhistidine, L-carnosine and DL-anserine were obtained from the California Foundation for Biochemical Research, Los Angeles.

Purification of carnosine–anserine synthetase

Ethanol step. The method described by Winnick & Winnick (1958, 1959*a*) was modified in two details. The minced pectoral muscle was homogenized in a Waring Blendor (220 v), instead of with a Potter homogenizer. The tissue was blended (at half the rated voltage), first for 1½ min. with 200 ml. of the buffer/100 g. of muscle, and then for another ½ min. with the addition of a second 200 ml. The other change was to fractionate the muscle extract between 20 and 60% (v/v) ethanol, instead of 25 and 65%.

Acetone step. As in the ethanol procedure, all operations were performed in a cold room (0–4°). The precipitate from the ethanol stage was dissolved in 0·04 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, pH 7·4, by using 130 ml. for each 100 g. of the original muscle. This solution (containing about 20 mg. of protein/ml.) was immersed in an ice–salt bath. Acetone, to a concentration of 28·5% (v/v), was added with stirring over a 10 min. period, during which the temperature of the solution fell to −5°. The precipitate was collected by centrifuging for 4 min. at 600 *g*. After draining off the supernatant phase as completely as possible, the precipitate was stirred briefly with a quantity of tris buffer equal to one-third of the original volume. The turbid suspension was centrifuged (3 min., 9000 *g*) to remove denatured protein. The resulting solution contained about 15 mg. of protein/ml. The next stage was performed without delay.

Calcium phosphate step. Again all operations were done in the cold. The method of Tiselius, Hjertin & Levin (1956)

* Part 3: Winnick & Winnick (1960).

was used to prepare calcium phosphate. The latter was suspended in mM-sodium phosphate (pH 6·8) and stored at 0°. Before use, it was centrifuged, washed with 0·04M-tris buffer, and suspended in this same medium at a concentration of about 120 mg. of calcium phosphate/ml. Two volumes of gel were added to the carnosine–anserine synthetase (CAS) solution derived from the previous step. After standing for 20 min., the suspension was centrifuged (3 min., 5000 *g*), and the supernatant solution was discarded. A volume of 0·01M-potassium phosphate (pH 7·4) equal to that of the original enzyme solution was added to the gel. The latter was stirred occasionally during a 20 min. period, and then centrifuged. The clear supernatant solution was retained. This eluate, containing approx. 0·3 mg. of protein and 0·04–0·06 enzyme unit/ml., was used in all experiments except when otherwise indicated.

Assay methods

Standard measurement of dipeptide synthesis. The method used (Winnick & Winnick, 1959*a*, 1960), was based upon the destruction by ninhydrin of excess of free [*carboxy*-^{14}C]-histidine (or histidine analogues) after incubation at 37°, and then measurement of radioactivity in the surviving [^{14}C]peptide. A slightly larger volume, 1 ml., was used for the incubation mixture, and, unless otherwise stated, the latter contained 2 μmoles of ATP, 2 μmoles of Mg^{2+} ions, 1 μmole of β-alanine, 1·3 μmoles of DL-[1-^{14}C]histidine, 0·02–0·03 unit of enzyme and 0·04M-tris buffer, pH 7·4. One unit of CAS activity is defined as the amount which synthesizes 1 μmole of dipeptide/hr.

Incorporation of [^{32}P]orthophosphate and pyrophosphate into adenosine triphosphate. The reaction mixture was that of the preceding paragraph, except that labelled histidine was replaced by radioactive phosphate (of specified molarity). After incubation for 1 hr. at 37°, the reaction was stopped by adding 1 ml. of 0·5M-perchloric acid. The precipitated protein was removed by centrifuging. To 1·5 ml. of the supernatant solution was added 0·2 ml. of acid-washed Norit A charcoal suspension (0·1 g./ml. of water). The mixture was stirred intermittently during 30 min. to facilitate adsorption of nucleotide. The charcoal was then sedimented and washed three times with 3 ml. of 0·05M-perchloric acid. It was transferred with 2 ml. of water to a steel planchet, and, after drying, the radioactivity was measured in a mica-window Geiger counter. Less than 0·05% of the initial inorganic ^{32}P was retained by the charcoal after the above treatment.

Other analyses. Protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Ribonucleic acid (RNA) was measured by the orcinol method of Drury (1948).

RESULTS

Purification of carnosine–anserine synthetase. The values given in Table 1 are typical of a large number of preparations made with 100–200 g. batches of chick pectoral muscle from 3–5-week-old chicks. Less than 0·5% of the original protein remained at the final stage, but a substantial part of the enzyme activity was retained. Assays of the crude extract were inaccurate, and the activity of the enzyme at this stage was probably depressed by product inhibition, i.e. by the high concentration of dipeptides present (Winnick & Winnick, 1959*a*). However, it can be concluded that about 100-fold purification was achieved. A number of other isolation techniques, including precipitation at acid pH, chromatography, electrophoresis, salt fractionation, precipitation with metals and various reagents, and different types of adsorbents and eluting agents, were all unsuccessful, owing in considerable degree to the marked instability of the enzyme. The addition of a variety of reagents (sulphydryl compounds, cyanide, sucrose, metal ions, different buffers, β-alanine, ATP) at different stages of the procedure had no favourable effect on the stability of the enzyme. While CAS at the ethanol stage could be stored for 1–2 weeks at −15° without significant inactivation, the acetone and calcium phosphate preparations lost much of their activity in a few days at −15°, and in 24 hr. at 0°.

Properties of the partially purified enzyme. The protein eluted from calcium phosphate gel was heterogenous when analysed by paper electrophoresis. It contained about 4% of ribonucleic acid, but there is no indication that the latter was a component of the enzyme molecule. As with the crude enzyme (Kalyankar & Meister, 1959), the partly purified CAS was not significantly inactivated by preincubation for 1 hr. at pH 7·0 and 25° with crystalline ribonuclease (20 μg./mg.).

The activity of the best preparations, about 0·2 unit/mg. of protein, is much lower than that of purified glutathione synthetase of yeast (Snoke, 1955) or wheat (Webster & Varner, 1955).

Most of the enzyme activity was lost upon freeze-drying or upon dialysis in ethylene diaminetetra-

Table 1. *Summary of purification of carnosine–anserine synthetase*

Stage	Total protein (mg./100 g. of muscle)	Total enzyme (units/100 g. of muscle)	Specific activity (unit/mg. of protein)
Initial muscle extract	6700	—	0·0006
Ethanol fraction	3900	10·6	0·0027
Acetone fraction	945	10·2	0·0109
Calcium phosphate eluate	26·5	3·5	0·132

acetic acid-treated cellophan tubing for several hours at 0°. After the latter operation, activity was not restored by adding back the external solution. Stability was slightly increased by the addition of serum albumin. The enzyme lost much of its activity within a few hours outside the pH range of 6–8 (even at 0°) or at higher salt concentrations (0·2 M-sodium chloride). It could not be precipitated from dilute solution by acetone, even when a protein carrier was used. The inability to concentrate the CAS solution without extensive loss in activity proved a serious disadvantage in certain experiments described below.

Requirements for optimum activity. Partly purified CAS, like the crude ethanol fraction (Winnick & Winnick, 1959*a*; Kalyankar & Meister, 1959), promoted maximum carnosine synthesis at pH 7·4 with approx. 1·0 μmole of L-histidine and 1–2 μmoles of β-alanine/ml. of incubation mixture, and with a linear reaction rate for the first hour at 37°. This stability at 37° is surprising, in view of the above-mentioned inactivation upon storage at low temperatures.

Fig. 1 shows a rather sharp peak for optimum carnosine synthesis at 2 mM-ATP. In previous studies, less pure CAS preparations showed a broader plateau from about 2 to 7 mM-ATP. This requirement for a higher concentration probably reflected adenylate kinase and adenosine triphosphatase activities. Adenosine diphosphate could replace ATP to a much smaller extent with the partly purified, as compared with the crude, CAS, which suggests a lower content of adenylate kinase in the calcium phosphate preparation. Also, triphosphates of guanosine, inosine, and cytidine were relatively less effective with the present enzyme.

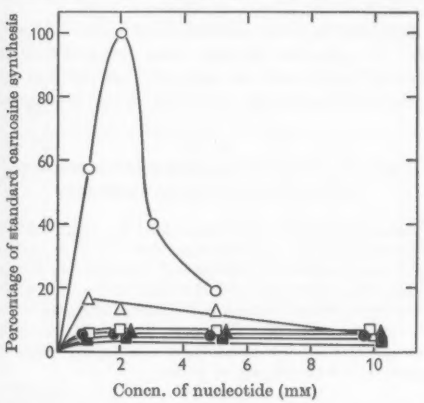

Fig. 1. Comparison of different nucleotides as activators of CAS. The values are expressed in terms of the standard assay with 2 mM-ATP, taken as 100. ○, ATP; △, ADP; □, uridine triphosphate; ●, guanosine triphosphate; ▲, inosine triphosphate; ■, cytidine triphosphate.

As in earlier studies, 2 mM-Mg^{2+} was required for optimum activity, and a higher concentration was inhibitory (Fig. 2). Mn^{2+} ions were able to substitute for Mg^{2+} ions to a considerable extent, and were even more effective at low concentration. These two metals exhibited the same relationships in the case of glutathione synthetase (Snoke, 1955). Other metallic ions (Fe^{2+}, Ni^{2+}, Mo^{2+}) were much less effective. Kalyankar & Meister (1959) reported that bivalent Mn^{2+} and Co^{2+} ions were less

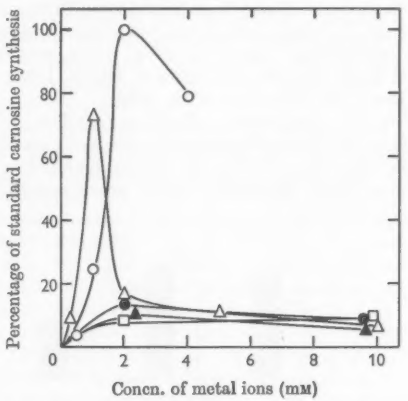

Fig. 2. Comparison of bivalent metal ions as substitutes for Mg^{2+} ions in dipeptide synthesis. The results are expressed in terms of the standard assay with 2 mM-Mg^{2+} taken as 100. ○, Mg^{2+}; △, Mn^{2+}; □, Fe^{2+}; ●, Ni^{2+}; ▲, Mo^{2+}.

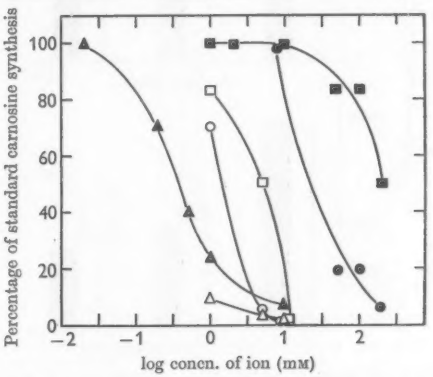

Fig. 3. Effects of various ions on CAS activity. Standard assay conditions were used (including 2 mM-Mg^{2+}). ○, Fe^{2+}; △, Ni^{2+}; □, Mo^{2+}; ■, K^{+}; ▲, Mn^{2+}; ●, F^{-}.

than 10% as active as Mg^{2+} ions with the ethanol preparation.

Fig. 3 shows that Mn^{2+} ions did not augment the action of Mg^{2+} ions, but inhibited the system at concentrations as low as 0·1 mM. It is difficult to reconcile this result with the finding that Mn^{2+} ions could partially replace Mg^{2+} ions as an activator of CAS. Ni^{2+} ions also inhibited strongly, Fe^{2+} and Mo^{2+} were less effective inhibitors, and fluoride exerted an effect only above 10 mM. In the case of K^+ ions, a mild inhibition of peptide formation was produced at between 10 and 100 mM. A slight stimulatory effect of potassium chloride at 150 μM in phosphate buffer has been observed with the crude enzyme (Kalyankar & Meister, 1959). By contrast, K^+ ions were found markedly to stimulate glutathione synthesis with either acetone-dried liver extracts (Johnston & Bloch, 1951) or purified yeast enzyme (Snoke, 1955).

Substrate specificity. Previous studies with various histidine analogues and other basic α-amino acids (Winnick & Winnick, 1958; Kalyankar & Meister, 1959) indicated a fairly broad specificity for crude chick-muscle CAS, and raised the possibility that more than one enzyme was present. Accordingly, it was of interest to test preparations at successive stages of purification, with respect to their rates of utilization of different histidine substrates for β-alanyl peptide formation. Fig. 4 shows that CAS at the calcium phosphate stage, unlike the other preparations, was relatively less active toward 1-methylhistidine, as compared with histidine. However, more information would be required in order to conclude that anserine formation is influenced by a separate synthetase enzyme. Actually, there is evidence that anserine can arise in muscle tissue by an independent pathway, namely the methylation of carnosine by *S*-adenosylmethionine (Winnick & Winnick, 1959*b*).

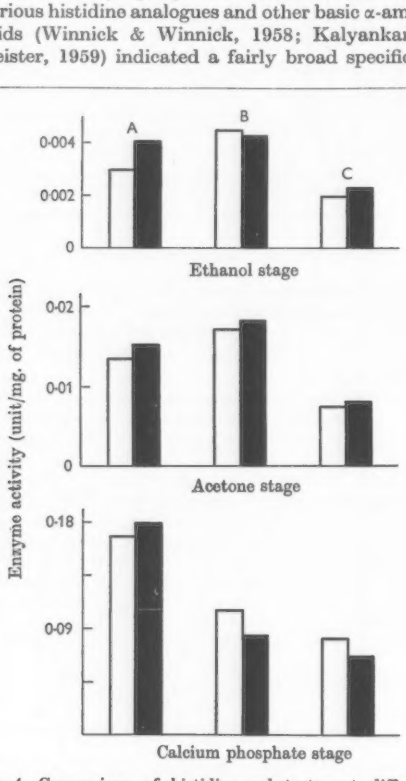

Fig. 4. Comparison of histidine substrates at different stages of purification of CAS. □, Concentration of 1·3 mM-; ■, 2·6 mM-labelled amino acid. A, Histidine; B, 1-methylhistidine; C, 1-ethylhistidine.

Transpeptidation. In connexion with the above question of the origin of anserine, it was desirable to test a third possible pathway: the transfer of a β-alanyl group from carnosine to methyl[^{14}C]-histidine. It can be seen in Table 2 that this reaction occurred to a much lesser extent than did the usual β-alanyl peptide synthesis. The transfer was still smaller when ethyl[^{14}C]histidine was used with carnosine, and zero when the analogous process with [^{14}C]histidine and anserine was tested. Results very similar to those of Table 2 were obtained with crude CAS. Unless different conditions or other enzymes are operative *in vivo*, it appears unlikely that transpeptidation represents a major pathway of anserine formation. It may be mentioned that purified yeast-glutathione synthetase promotes an exchange of labelled glycine with the glycine of the tripeptide, in the presence of Mg^{2+} ions, ATP and arsenate (Snoke & Bloch, 1954). In this case the ratio of transfer to synthesis is 1:11.

Experiments on the mechanism of carnosine formation. In previous studies with crude CAS, the working hypothesis was adopted that the first step in carnosine synthesis consisted in the interaction

Table 2. *Attempted transpeptidation reactions with carnosine–anserine synthetase*

The assay involved incubation for 1 hr. at 37° of 0·02-0·03 unit of purified CAS with 2 μmoles of ATP, 2 μmoles of Mg^{2+} ions, 2 μmoles of peptide and 1·5 μmoles of labelled amino acid, all in 1 ml. of 0·04 M-tris buffer (pH 7·4). The ninhydrin method (Winnick & Winnick, 1960) was used to measure formation of [^{14}C]peptide. The units are defined similarly to those for carnosine synthesis, for which the activity was 0·15 unit/mg. of protein.

Isotopic amino acid	Peptide	Transfer activity (unit/mg. of protein)
DL-Methyl[^{14}C]histidine	L-Carnosine	0·010
DL-Ethyl[^{14}C]histidine	L-Carnosine	0·002
DL-[^{14}C]Histidine	DL-Anserine	0·000

of β-alanine, ATP and enzyme (E) to yield an activated form of the amino acid, with pyrophosphate as a by-product:

$$\beta\text{-Alanine} + \text{ATP} + \text{E} \overset{Mg^{2+}}{\rightleftharpoons} \text{E–}\beta\text{-alanine–AMP} + \text{pyrophosphate}$$

The second stage would be:

$$\text{E–}\beta\text{-alanine–AMP} + \text{L-histidine} \rightleftharpoons \text{L-carnosine} + \text{E} + \text{AMP}$$

The first phase corresponds to the much-studied process of α-amino acid activation. Some support for the involvement of pyrophosphate was the strong inhibition produced by this compound (as compared with orthophosphate) on β-alanyl peptide synthesis with crude enzyme. Fig. 5 gives very similar results for partly purified CAS.

An excess of adenosine 5′-phosphate (AMP) also depressed carnosine formation (Fig. 5), and ADP exerted an even stronger effect. It has already been mentioned (Fig. 1) that high concentrations of ATP inhibited carnosine synthesis. These effects of adenine nucleotides cannot at present be explained.

An indication that the overall reaction is not readily reversed was provided by incubating radioactive (tritiated) carnosine with CAS in the presence of various combinations of AMP, ATP, pyrophosphate, β-alanine and histidine. In all cases less than 1 % of the total isotope was found in the β-alanine region of chromatograms. In these experiments, histidine was not resolved from the carnosine area, and the latter accounted for 98–99 % of the tritium.

The incorporation of [^{32}P]pyrophosphate into ATP, commonly used to characterize the α-amino acid-activation process, could not be tested with crude CAS, because of a high exchange rate which was independent of added β-alanine (Kalyankar & Meister, 1959; Winnick & Winnick, 1960). After considerable purification, the enzyme still promoted a moderate degree of [^{32}P]pyrophosphate incorporation in the absence of β-alanine. In certain experiments, as in Fig. 6, a low β-alanine concentration slightly stimulated the reaction, but this effect was not consistently reproducible and was probably not outside experimental error. [^{32}P]Orthophosphate was not appreciably incorporated into ATP.

Other experiments bearing on this question, for which data are not presented, can be mentioned briefly. Preincubation of the enzyme with ATP and histidine, in order to use up traces of β-alanine that might conceivably have been present, failed to improve subsequently the β-alanine dependence of the [^{32}P]pyrophosphate exchange reaction. Actually, no β-alanine could be detected upon analysis of acid extracts of the enzyme. At acid pH (5·0 and 6·5) the incorporation of pyrophosphate into ATP was very low, and again it was not stimulated by β-alanine. In addition γ-aminobutyric acid, an analogue of β-alanine, was not effective (at pH 7·4).

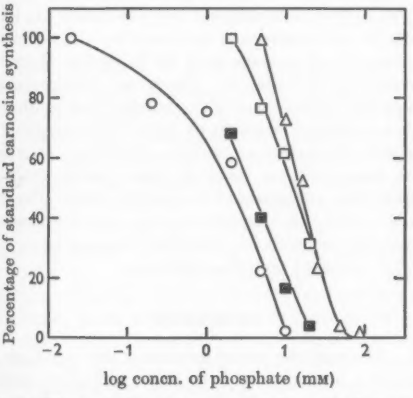

Fig. 5. Effect of phosphate compounds on CAS activity. The values are in terms of standard assay conditions, with 2 mM-ATP present. The orthophosphate concentration includes that present in the added enzyme solution. ○, Pyrophosphate; △, orthophosphate; □, AMP; ■, ADP.

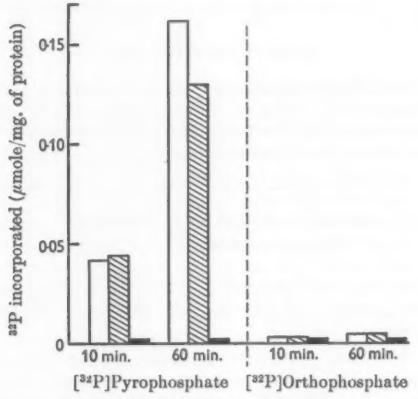

Fig. 6. Incorporation of [^{32}P]orthophosphate and [^{32}P]-pyrophosphate into ATP. The complete mixture (□) contained, in addition to CAS: 1 mM-β-alanine, 2 mM-ATP, 2 mM-Mg^{2+}, and 1 mM-labelled phosphate. ▨, β-Alanine omitted; ■, enzyme omitted. The activities are expressed per mg. of enzyme protein.

Very high concentrations of β-alanine strongly inhibited the [^{32}P]pyrophosphate exchange reaction (Fig. 7). By contrast, α-alanine had no effect on this process.

Table 3 shows that high concentrations of fluoride likewise inhibited pyrophosphate–ATP exchange. This blockage also occurred in the presence of a large excess of β-alanine. The complete inhibition of the overall reaction by potassium fluoride resembles the results of Davis & Novelli (1958) with extracts of peas. They found that elevated fluoride concentration strongly depressed both endogenous and α-amino acid-dependent [^{32}P]pyrophosphate–ATP exchange.

In the present research, experiments were also performed with ^{14}C-labelled ATP and with ATP labelled in the terminal phosphate with ^{32}P, in attempts to correlate peptide-bond formation with hydrolysis of ATP, as was done for glutathione synthesis (Snoke & Bloch, 1954). There was some indication that low concentrations of β-alanine stimulated ATP breakdown in the presence of CAS and histidine, but the available enzyme solutions were too dilute for accurate measurement of the effect.

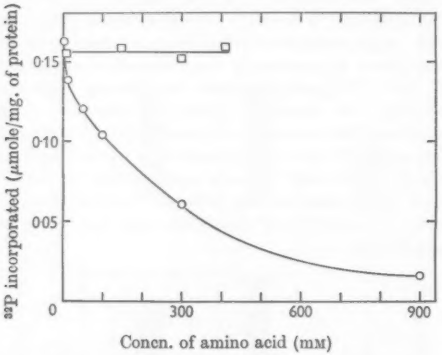

Fig. 7. Effect of high alanine concentrations on [^{32}P]pyrophosphate–ATP exchange. The incubation mixture contained 2 mM-ATP, 2 mM-Mg^{2+}, 2 mM-[^{32}P]pyrophosphate, and enzyme. ○, β-Alanine; □, α-alanine.

Table 3. *Inhibition by fluoride of [^{32}P]pyrophosphate–ATP exchange*

The assay conditions were those of Fig. 7, except that 2 mM-β-alanine and various concentrations of KF were present.

Concentration of fluoride (mM)	Exchange activity (μmole of [^{32}P]pyrophosphate incorporated into ATP/mg. of protein)
0	0·125
10	0·095
50	0·069
100	0·006
200	0·000

As in the case of crude CAS (Winnick & Winnick, 1960), attempts to demonstrate the formation of β-alanine hydroxamate, or an adenylate of the β-amino acid (by charcoal adsorption or paper chromatography methods), were unsuccessful. Likewise, it was not possible to detect an activated form of the enzyme, after incubation with β-alanine plus [^{14}C]ATP, or with [^{14}C]-β-alanine and ATP. Previous experiments with synthetic β-alanine adenylate (Winnick & Winnick, 1960) were complicated by the instability of this compound, and its ability to combine non-enzymically with histidine. However, it is of interest that Kalyankar & Meister (1959) have been able to obtain an enzymic synthesis of ATP from [^{32}P]pyrophosphate and β-alanine adenylate in the presence of Mg^{2+} ions and fluoride.

Three types of evidence in the present paper support the view that the observed pyrophosphate–ATP exchange was an integral part of the overall process catalysed by CAS: (1) pyrophosphate is a strong inhibitor of carnosine synthesis; (2) fluoride inhibits both the pyrophosphate–ATP exchange reaction and carnosine formation; and (3) the enzyme catalyses the exchange process at maximum rate in the absence of detectable amounts of β-alanine. This last finding is analogous to the observation made by Novelli (1959) with a glycine activating enzyme of *Photobacterium fischeri*. This enzyme promoted a vigorous glycine-independent incorporation of orthophosphate into ATP.

The above facts suggest the possibility that the initial step in carnosine synthesis may involve the interaction of enzyme and ATP, rather than the activation of β-alanine. Such an interpretation would be in agreement with the observed inhibition of the exchange reaction by high concentrations of β-alanine (but not α-alanine). However, it is felt that the available data do not justify detailed speculation on alternative mechanisms. Further studies with more active enzyme preparations are obviously required for the elucidation of the pathway of β-alanyl peptide synthesis.

SUMMARY

1. The enzyme which promotes the synthesis of carnosine and anserine from their component amino acids has been purified about 100-fold, from an extract of chick pectoral muscle. The successive steps were fractionation with ethanol, followed by acetone, and then adsorption and selective elution of the enzyme from calcium phosphate gel. The

purification experiments were severely hampered by the lability of the enzyme.

2. Peptide-bond formation specifically required adenosine triphosphate, and magnesium ions could be replaced to a considerable extent by manganous ions. Carnosine synthesis was not readily reversible. Pyrophosphate was strongly inhibitory, and orthophosphate less so. Although the enzyme preparation promoted [^{32}P]pyrophosphate incorporation into adenosine triphosphate, a dependency upon β-alanine could not be clearly demonstrated. In fact, this process was suppressed by a high β-alanine concentration. Fluoride ions inhibited both carnosine synthesis and pyrophosphate–adenosine triphosphate exchange.

3. Attempts to detect an activated intermediate stage in carnosine synthesis were not successful.

4. β-Alanyl transfer from carnosine to 1-methylhistidine to yield anserine proceeded very slowly, as compared with direct dipeptide synthesis by the enzyme.

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REFERENCES

Davis, J. W. & Novelli, G. D. (1958). *Arch. Biochem. Biophys.* **75**, 299.
Drury, H. F. (1948). *Arch. Biochem.* **19**, 455.
Johnston, R. B. & Bloch, K. (1951). *J. biol. Chem.* **188**, 211.
Kalyankar, G. D. & Meister, A. (1959). *J. biol. Chem.* **234**, 3210.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Novelli, G. D. (1959). *Biochim. biophys. Acta*, **33**, 261.
Snoke, J. E. (1955). *J. biol. Chem.* **213**, 813.
Snoke, J. E. & Bloch, K. (1954). In *Glutathione*, p. 129. Ed. by Colowick, S. P. *et al.* New York: Academic Press Inc.
Tiselius, A., Hjertin, S. & Levin, O. (1956). *Arch. Biochem. Biophys.* **65**, 132.
Webster, G. C. & Varner, J. E. (1955). *Arch. Biochem. Biophys.* **55**, 95.
Winnick, R. E. & Winnick, T. (1958). *Bull. Soc. Chim. biol., Paris*, **40**, 1727.
Winnick, R. E. & Winnick, T. (1959*a*). *Biochim. biophys. Acta*, **31**, 47.
Winnick, R. E. & Winnick, T. (1960). *Biochim. biophys. Acta*, **37**, 214.
Winnick, T. & Winnick, R. E. (1959*b*). *Nature, Lond.*, **183**, 1466.

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The Fractionation of Urine Colloids on Anion-Exchange Cellulose

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Urine contains a complex mixture of high-molecular-weight material, some components of which may be derived from the serum through a process of glomerular filtration or secretion and some of which may be derived from the urinary tract itself. Information about the nature of the urine colloids, however, has lagged considerably behind the knowledge of those in serum. Three main classes of high-molecular-weight compounds in urine have been recognized: proteins, glycoproteins and mucopolysaccharides. The presence of protein in urine was recognized as early as 1895 by Möerner and urine proteins have since been studied extensively by many workers, particularly Rigas & Heller (1951) and McGarry, Schon & Rose (1955). The variation in the amount of heat-coagulable protein in urine with several diseases, particularly the nephrotic syndrome, is well known (Heremans, 1958).

The term 'glycoprotein' is used to indicate components containing appreciable amounts of hexosamine, which can be liberated only after vigorous hydrolysis. Glycoproteins generally also contain hexose and sialic acid, with little or no uronic acids. Urinary fractions presumed to contain glycoproteins have been studied by Boyce, King, Little & Artom (1958), Boyce & King (1959) and King, Boyce, Little & Artom (1958). One normal urinary glycoprotein, first described by Tamm & Horsfall (1950), has been extensively characterized with respect to its composition and physical properties (Tamm & Horsfall, 1952; Tamm, Bugher & Horsfall, 1955; Porter & Tamm, 1955). Anderson (1954) has reported the isolation of two heterogeneous glycoprotein fractions from normal urine. Application of a method for estimating one of these fractions (Anderson & Maclagan, 1955)

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showed considerable variation from normal in a number of diseases (Lockey, Anderson & Maclagan, 1956). This fraction was found to possess a wide range of biological properties, including antitryptic, gonadotrophic, amylase and vitamin B_{12}-binding activities, and to be a potent inhibitor of viral haemagglutination (Anderson, Lockey & Maclagan, 1955). In addition, the presence of urokinase and uropepsinogen activities has been observed (Kickhöfen, Struwe, Bramesfeld & Westphal, 1958) in this fraction. The fact that similar biological properties have been reported for serum glycoprotein fractions suggests that some of the urine glycoproteins may be derived from serum.

Mucopolysaccharides, such as chondroitin sulphate, also occur in urine. These may occur free of protein or may exist as mucopolysaccharide–protein complexes similar to those observed in cartilage by Shatton & Schubert (1954). There have been several reports of the presence of these colloids in normal urine (Rich & DiFerrante, 1956; DiFerrante & Rich, 1956; Jacques, Bapke & Levy, 1953; Kerby, 1954), and of their elevated levels in several diseases (Craddock & Kerby, 1955).

Several reports are available on the nature and amount of the total colloids both in normal urine and various pathological conditions (Hammerman & Hatch, 1955; King *et al.* 1958; Maclagan & Anderson, 1958*a*; Björnesjö, Werner & Odin, 1959). Electrophoretic and chemical studies have been carried out on several heterogeneous urine-colloid fractions obtained after salt-fractionation procedures (Boyce, Garvey & Norfleet, 1954; Boyce *et al.* 1958; Boyce & Swanson, 1955; Boyce & King, 1959; King *et al.* 1958; King, Little, Boyce & Artom, 1959). It is likely that a more complete fractionation and characterization of the high-molecular-weight components in urine will be followed by valuable information about their source, physiological significance and clinical importance.

This paper describes the fractionation of non-ultrafiltrable, non-dialysable urine components on cellulose ion-exchange columns and a study of some of the chemical and physical properties of some of the fractions so separated. An investigation of the ability of these fractions to bind calcium is also included, together with the calcium-binding ability of the colloids in complete 24 hr. specimens of both normal and pathological urines.

METHODS

The methods used in these studies included the following. Urine glycoproteins: Anderson & Maclagan (1955); sialic acid: Werner & Odin (1952); hexose: Winzler (1955); hexosamine: Rondle & Morgan (1955); fucose: Dische & Shettles (1948); nitrogen: micro-Kjeldahl; calcium: Ferro & Hamm (1957); hexuronic acid: Dische (1947); protein: Lowry, Rosebrough, Farr & Randall (1951); qualitative and quantitative precipitin reactions: K..bat & Meyer (1948); immunoelectrophoresis: Williams & Grabar (1955); paper electrophoresis: Durram (1950), Flynn & De Mayo (1951); chloride: by a method involving the polaragraphic estimation of residual silver ions when a chloride-containing solution is added to silver nitrate in 0·1 N-nitric acid.

Inhibition of virus haemagglutination. Inhibitor activity was determined with Lee B influenza virus harvested from allantoic fluid and converted into the indicator phase by heating at 56° for 30 min. Serial twofold dilutions of the virus preparation were made in 5 mM-phosphate buffer, pH 7·0, and to 0·5 ml. of each dilution was added 0·25 ml. of 0·5% suspension of washed, type-O human erythrocytes. After mixing, the tubes were incubated at 4° overnight. The last dilution of the virus to show agglutination was taken as 1 haemagglutination (HA) unit. Serial twofold dilutions were made from portions of the fractions taken from the DEAE-cellulose column, and to 0·25 ml. samples of these dilutions were added 0·25 ml. of virus suspension containing 8 haemagglutinating units of the indicator virus. After incubation for 4 hr. at 4°, 0·5 ml. of a 0·5% suspension of erythrocytes was added, and the tubes were read after incubation at 4° overnight. The last dilution to inhibit haemagglutination was defined as 1 inhibiting (HAI) unit. The specific activity of each fraction was expressed as HAI units/μg.

Determination of calcium-binding. Calcium-binding was determined by an equilibrium-dialysis technique in which a known amount of the colloid under investigation (about 10 mg.) was dissolved in 11 ml. of veronal buffer (0·01 M, pH 9·0) and dialysed against 20 ml. of the veronal buffer containing 5 mM-calcium chloride. After equilibration by slow shaking overnight at 4°, the concentrations of calcium and chloride (μequiv./ml.) both inside and outside the dialysis sac were determined. The amount of bound calcium inside the sac was then calculated, assuming the existence of the simple Donnan equilibrium, the amount of calcium bound by the colloid fraction being expressed as μequiv. of calcium/mg. of colloid. The same method was used for estimating the calcium-binding by urine specimens, 100 ml. of the 24 hr. urine output first being dialysed, freeze-dried and then taken up in 11 ml. of the veronal buffer. In this case results were expressed as the amount of calcium (μequiv.) bound by the colloids in the complete 24 hr. urine specimen.

Ultrafiltration of the urine colloids. Urine, preserved with thymol, was stored for 24 hr. at 4° and insoluble material removed by filtration. The urine was then ultrafiltered in 4 l. batches under air pressure at 65 lb./in.^{2} at 4° through collodion membranes of diam. 15·5 cm. (Schleicher and Schüll, L.S.G. 60) capable of retaining spherical molecules of molecular weights of 10 000 and over. The used membranes were immediately stored at $-20°$.

Separation of the urine colloids from the membranes. In a typical experiment the colloids obtained from 16·6 l. of pooled normal male urine were extracted from the membranes by soaking them three times for 24 hr. at 4° in 100 ml. of the starting buffer (SB) described in Table 2. The pooled washings were dialysed at 4° against running distilled water for 72 hr. and were freeze-dried, yielding 960 mg. of fraction A (58 mg./l.). The membranes were

then shredded and dissolved in 300 ml. of an ethanol–ether mixture (1:1) at 4°; the solution was mixed with an equal volume of 0·1 N-NaOH, filtered and the ether removed by aeration. The solution was dialysed at 4° for 72 hr. against running distilled water, and freeze-dried to yield 2340 mg. of fraction B (141 mg./l.). An additional 12 mg./l. did not dissolve in 0·1 N-NaOH after dissolution of the membranes. The protein and carbohydrate composition of fractions A and B is shown in Table 1.

The composition of these fractions suggests the presence of considerable amounts of carbohydrate-rich components, as previously found by Hammermann & Hatch (1955), Boyce *et al.* (1954), Boyce & King (1959), King *et al.* (1958, 1959) and others. The differences in the composition of fractions A and B may in part be due to molecular size, with relatively more of fraction B being in the interstices of the collodion membrane, and in part to the solubility properties, fraction B being dissolved in 0·1 N-NaOH and fraction A in the starting buffer at pH 9·0. For subsequent fractionation the non-dialysable residues A and B were combined.

Fractionation of the urine colloids. The procedure developed by Sober, Cutter, Wyckoff & Peterson (1956) was employed, the anion-exchange cellulose adsorbent diethylaminoethylcellulose (DEAE-cellulose) being used.

DEAE-cellulose (80–140 mesh, 0·71 m-equiv. of ionizing groups/g., obtained from Brown Co., Berlin, New Hampshire, U.S.A.) was washed successively with N-NaOH, N-HCl and N-NaOH, filtration under suction being used each time. After washing to neutrality with water, it was then washed with the starting buffer described in Table 2. The exchanger was then poured as a slurry into a glass tube (length 42 cm., diam. 2·8 cm.), the lower end of which contained a layer of glass wool supported on a coarse metal grid. After the cellulose had settled by gravity the column was further compacted by application of air pressure at 5 lb./in.^{2} By this means, 40–50 g. of cellulose exchanger formed a packed column 35–40 cm. high. Several litres of the starting buffer were then passed through the column. The flow rates under gravity and under air pressure of 5 lb./in.^{2} were 0·74 and 3·3 ml./min. respectively.

Urine colloids (1–3 g.) were suspended in about 25 ml. of starting buffer, and the mixture was stirred continuously for 18 hr. at 4°. Insoluble material was centrifuged off and resuspended in fresh buffer, stirring as before, this process being repeated three times. The combined supernatant fluids contained 70% of the total non-dialysable residue. This solution was then applied to the top of the column and allowed to enter under gravity, and was washed in with about 20 ml. of starting buffer.

A pH and ionic-strength gradient was then applied to the column, the Varigrad mixing device developed by Peterson & Sober (1958) being used. This consisted of nine cylinders (designated 1–9) each 7·5 cm. in diam. and 13 cm. high. These were connected at their bases in series, the outlet from cylinder 1 leading to the top of the cellulose column via a pumping device through which the eluting buffers were supplied. Cylinders 1–9 contained buffers of decreasing pH and increasing ionic strength.

A 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–citrate buffer system was devised, for use with the Varigrad apparatus, which would deliver buffer to the cellulose column at a continuous pH descent from 9 to 3, accompanied by an increasing ionic strength. The compositions of the starting buffer, limit buffer and the Varigrad mixtures are shown in Table 2. After about 3 l. had passed through the column, three further eluting solutions were used (buffers 2–4), the final effluent emerging at a pH of 1·5. [Buffer 2 (pH 2·25): tris, 30·54 g.; citric acid, 205·0 g.; sodium chloride, 5·85 g.; water, 750 ml. Buffer 3 (pH 1·46): citric acid, 210·14 g.; water, 1000 ml. Buffer 4 (pH 1·41): citric acid, 210·14 g.; sodium chloride, 58·5 g.; water, 1000 ml.] At pH values below 2·0 considerable retraction of the cellulose occurred, necessitating a gradually increasing air pressure up to a maximum of 80 lb./in.^{2} in order to maintain a conveniently rapid rate of flow. The effluent was collected in individual volumes of 10 ml., with a fraction collector, the total volume being about 4 l. The complete chromatographic separation was accomplished in 24–30 hr.

The fractions from the column were examined in a Beckman spectrophotometer at 280 mμ. The fractions comprising the various peaks were then combined, filtered, dialysed against running distilled water at 4° for 3 days and were freeze-dried.

RESULTS

Normal urine

Fig. 1 shows a chromatogram of the urine colloids from 13·3 l. of pooled normal male urine (1860 mg.), isolated as described above. A number of poorly resolved peaks are evident.

Table 1. *Composition of some components in the non-dialysable fractions A and B*

	Non-dialysable residue A (%)	Non-dialysable residue B (%)
Sialic acid	6·2	1·0
Hexose	8·4	39·0
Hexosamine	15·8	2·0
Hexuronic acid	1·4	4·4
Protein	37·0	14·0

Table 2. *Buffer system used for the separation of urine colloids on DEAE-cellulose column*

Buffer 1: starting buffer (SB) contained (in 1 l. of water): tris, 24·429 g.; citric acid, 1·0507 g. Limit buffer (LB) contained (in 5 l. of water): tris, 122·14 g.; citric acid, 315·21 g.; sodium chloride, 5·85 g. The table shows the compositions of buffers used for the Varigrad apparatus. Buffers 2–4 are described in the text.

Cylinder no.	SB (ml.)	LB (ml.)	pH
1	400·0	0	9·00
2	397·5	2·5	7·89
3	397·5	2·5	7·89
4	393·0	7·0	5·46
5	393·0	7·0	5·46
6	393·0	7·0	5·46
7	385·0	15·0	4·30
8	150·0	250·0	3·01
9	0	400·0	2·79

The composition of the fractions, pooled as indicated, is given in Table 3. Only about 38% of the colloids placed on the column could be recovered in the effluents, indicating that a considerable amount of irreversible absorption on to the DEAE-cellulose had occurred. The protein percentages obtained by the method of Lowry *et al.* (1951) correlated fairly well with the amount of light-absorption at 280 mμ (Fig. 1). Both these procedures depend primarily on the presence of aromatic amino acids. Protein percentages calculated from the total nitrogen values, however, were consistently higher, particularly for fractions 10, 14 and 15, indicating that these fractions were probably deficient in aromatic amino acids. It would therefore appear that protein percentages calculated from the total nitrogen figures are a more reliable index of true protein content. Each of the fractions showed the presence of considerable amounts of carbohydrate. Fractions 1, 2 and 9 were characterized by being especially rich in fucose, fractions 9, and 12–15 in hexuronic acid and 6, 9–10 in sialic acid.

Although the hexose:hexosamine:sialic acid ratios of fractions 1–8 varied somewhat, in general they approximated more closely to those that have been reported for the serum proteins (Winzler, 1955; Sonnet, 1956; Laurent, 1958) than did the corresponding ratios for fractions 9–15. The higher proportions of hexuronic acid in the later fractions indicated an elution of acid mucopolysaccharides. Non-dialysable calcium was associated with all fractions but especially in the later fractions, with the exception of fraction 12. Fractions 9, 10 and 11, showing little absorption at 280 mμ, contained 62% of the calcium eluted from the column.

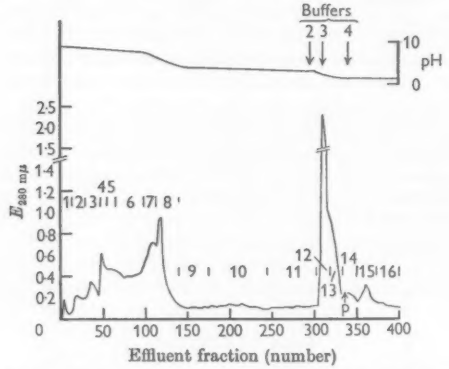

Fig. 1. Chromatogram obtained from the fractionation of the colloids from normal male urine on DEAE-cellulose. 'p' indicates a fraction that precipitated spontaneously from solution immediately after emerging from the column.

Table 3. *Percentage composition of fractions from pooled normal male urine obtained by elution from DEAE-cellulose column*

Percentage concentration of proteins (column 11) was obtained in two ways: (*a*) calculated by multiplying the total nitrogen, after subtracting hexosamine N (7·8%) and sialic acid N (4·54%), by 6·25; (*b*) by the method of Lowry *et al.* (1951).

Fraction no.	Yield (mg.)	Conen. in effluent (mg./ml.)	Hexose (%)	Hexos-amine (%)	Sialic acid (%)	Fucose (%)	Hex-uronic acid (%)	Calcium (%)	Total nitrogen (%)	Proteins (%) (*a*)	Proteins (%) (*b*)	Calcium-binding (μequiv. of Ca/mg. of colloid)	Haemagglutination inhibition (HAI units/mg.)
1	41·8	0·53	9·6	6·33	3·66	2·73	1·31	0·34	—	—	—	0	0
2	22·0	0·14											0
3	23·5	0·13											0
4	36·4	0·33											0·024
5	53·8	0·26	10·7	6·7	5·36	1·42	0·90	0·62	10·90	63·6	54·0	0·155	
6	53·3	0·29	10·0	7·2	5·67	1·02	1·53	0·72	10·20	58·8	43·6	0·016	0·25
7	68·5	0·60	13·3	9·3	8·53	1·73	0·80	0·86	—	—	58·7	0	1·8
8	75·2	0·29	6·8	5·0	3·76	0·79	0·84	0·72	13·0	78·0	52·8	0·221	0·43
9	28·4	0·08	8·4	5·3	5·28	0·93	1·14	0·92	10·9	64·3	48·5	0·385	0·44
10	120·8	0·19	12·5	9·1	8·72	3·60	2·26	2·58	8·66	47·7	31·6	0·189	0·38
11	67·8	0·12	7·5	7·4	8·68	0·77	1·35	4·07	9·93	56·4	20·2	0·637	0·68
12	44·4	0·34	4·8	3·1	3·22	0·47	1·23	3·31	—	—	18·1	0·263	0·067
13	32·3	0·14	16·8	5·9	5·56	1·34	3·06	0·46	10·54	61·7	41·2	0·249	3·30
14	21·8	0·14	11·4	3·4	2·30	1·15	2·60	2·72	9·44	56·7	32·4	0·737	0
15	20·0	0·12											0
			11·4	4·6	1·68	1·48	5·06	2·88	7·54	44·5	18·2	0·880	0·27
Total:	710·0												

All of the fractions obtained were obviously extremely heterogeneous. This is evident in Fig. 2, which shows the results from paper electrophoresis of fractions 1–8 at pH 8·6 and 4·6.

At pH 8·6 these fractions showed a gradual increase in electrophoretic mobility, predictable from their behaviour on the ion-exchanger, protein eluting first having the lowest negative charge. Fractions 1–8 gave an intense stain with bromophenol blue, whereas fractions 9–15 stained feebly. Fractions 9–12 had mobilities mainly of α- and β-globulins whereas fractions 13 and 15 were immobile.

At pH 4·6 increasing amounts of negatively charged material staining with bromophenol blue were present in fractions 1–8. Of the feebly staining fractions 9–15, fraction 12 had negatively charged material which migrated considerably further than human-serum orosomucoid, indicating the presence in urine of colloids which so far have not been reported present in serum.

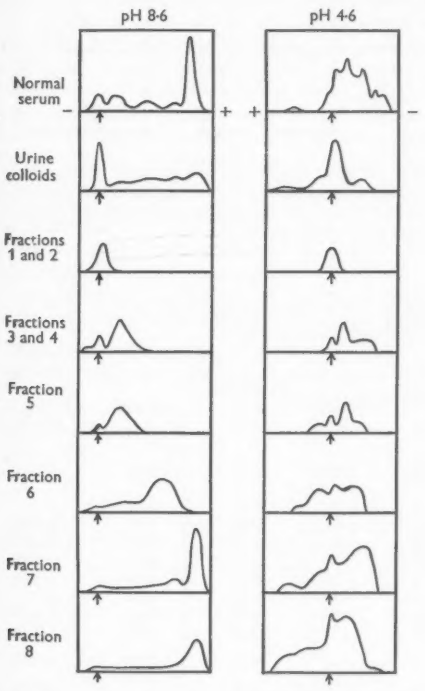

Fig. 2. Electrophoresis patterns of the fractions obtained from the separation of normal urine colloids on DEAE-cellulose. Paper electrophoresis was carried out at pH 8·6 and pH 4·6.

Some biological activities of the urine-colloid fractions

Inhibition of viral haemagglutination. Human urine is known to be a rich source of an inhibitor of influenza viral haemagglutination. Fractions high in inhibitory activity have been isolated and studied by Tamm & Horsfall (1950, 1952) by Faillard (1956) and by Anderson *et al.* (1955) among others. The urine fractions from the DEAE-cellulose column were therefore examined for their abilities to inhibit viral haemagglutination. The dialysed effluents from the column before freeze-drying were used, the colloid concentrations present being listed in Table 3 (column 3).

The last column of Table 3 shows the specific activities of the various fractions. The distribution clearly indicates the presence of several areas of activity with the highest specific activities found in fractions 6 and 12. These fractions had activities comparable with that of the glycoprotein isolated by Tamm & Horsfall (1950).

Antigenic components. Antibodies prepared in chickens to the uromucoid of Boyce & Swanson (1955), and tested by the method of Goodman *et al.* (1957), were found to react strongly with fractions 4–8 and also with 12 and 15. This suggests that the uromucoid fraction is quite heterogeneous or that the processes of fractionation resulted in a separation into degraded products or both.

Fractions 6–8, particularly the last, reacted with antisera prepared in chickens to human-serum albumin and human-serum orosomucoid, which indicated that these fractions contained these serum proteins.

Immunoelectrophoretic analyses showed the presence of one or more arcs when fractions 2–8 from pathological urine II (see below) reacted with anti-human serum. Other fractions had no arcs or very weak ones. This evidence again suggests that fractions 2–8 contain some of the proteins derived from serum.

Pathological urine I

Urine from a patient who was severely paralysed with poliomyelitis and forming bilateral renal calculi was studied. There was no evidence of proteinuria by the heat and acetic acid methods. Significant bacteriuria with a mixture of *Escherichia coli*, *Proteus mirabilis* and enterococci was present.

A volume (14·4 l.) of the urine was treated as described previously. The yield of colloids soluble in the starting buffer placed on the column was 2550 mg. (177 mg./l.). These colloids were fractionated on a freshly prepared DEAE-cellulose column under the same conditions as were used for the normal urine. The chromatogram obtained is shown in Fig. 3. The distribution of components was rather similar to that shown in Fig. 1 for

normal urine, but the proportions in the different peaks were somewhat different.

The analytical results for the fractions are presented in Table 4. The ratios of hexose:hexosamine:sialic acid were considerably different from those for normal urine, primarily as a result of lower sialic acid and hexosamine contents and a higher hexose content. Fucose content was also below the values found in the normal urine.

As with the normal fractions, increased amounts of acid mucopolysaccharide were eluted from the

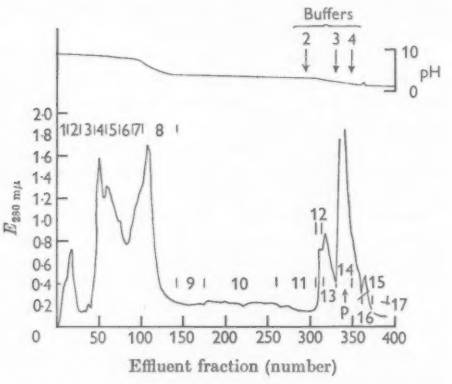

Fig. 3. Chromatogram of the colloids from a stone-forming poliomyelitis urine obtained after fractionation on DEAE-cellulose. 'p' indicates a fraction that precipitated spontaneously from solution immediately after emerging from the column.

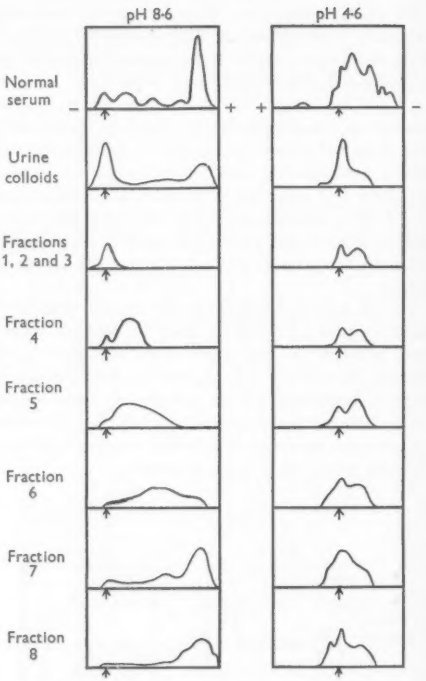

Fig. 4. Electrophoresis patterns of the fractions obtained from the separation of urine colloids from a stone-forming poliomyelitis urine. Paper electrophoresis was carried out at pH 8·6 and pH 4·6.

Table 4. *Percentage composition of fractions from poliomyelitis urine I obtained by elution from DEAE-cellulose column*

Fraction no.	Yield (mg.)	Concn. in effluent (mg./ml.)	Hexose (%)	Hexosamine (%)	Sialic acid (%)	Fucose (%)	Hexuronic acid (%)	Calcium (%)	Calcium-binding (μequiv. of Ca/mg. of colloid)
1	11·7	0·08							
2	2·8	0·01	13·8	7·55	2·34	1·86	1·02	2·42	0·252
3	7·6	0·05							
4	97·8	0·51	13·3	4·87	2·65	0·74	0·51	0·23	0
5	66·5	0·35	13·2	3·75	1·34	0·64	0·83	1·06	0·148
6	81·8	0·38	13·5	3·36	1·59	0·78	1·31	1·21	0·595
7	192·7	0·67	7·2	1·45	0·97	—	0·51	0	0·068
8	104·8	0·31	9·0	2·13	1·14	0·58	0·91	1·21	0·285
9	32·3	0·10	—	—	1·30	—	1·32	4·54	0
10	62·8	0·07	14·0	3·83	0·91	2·38	1·85	6·36	0·447
11	43·3	0·07	11·3	4·93	0·81	—	2·21	6·73	0·275
12	14·6	0·14	—	7·98	1·55	—	1·41	2·50	0·053
13	4·1	0·03							
14	104·3	0·54	13·1	3·61	2·04	1·76	2·88	0	0·094
15	36·5	0·22	23·8	3·00	1·07	—	1·67	0·91	0·366
16	31·3	0·07	12·1	3·78	0·51	1·20	4·72	9·16	0·164
17	20·7	0·06							
Total:	915·6								

column at higher ionic strengths and lower pH values; 48% of the total non-dializable calcium was located in fractions 9, 10 and 11.

Fig. 4 shows the results obtained by paper electrophoresis of these fractions at pH 8·6 and 4·6. Electrophoretic mobilities and staining properties were rather similar to those obtained for the normal urine fractions. At pH 4·6, however, less negatively charged material was present, indicating a lower acid glycoprotein content than in normal urine. This conclusion is in accord with the lower sialic acid values shown in the analytical figures in Table 4.

Reactions against antibodies to human-serum albumin were positive for fractions 6, 7 and 8 only. Reactions with anti-orosomucoid serum were positive for fractions 6 and 7, as was the case for the fractions from normal urine. In addition a strong reaction was given by fraction 4.

Pathological urine II

This urine was from a patient severely paralysed by poliomyelitis but who at the time of urine collection had never formed stones. No clinical proteinuria was present, although the urine was infected with *Aerobacter aerogenes*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and a species of *Enterococcus*. The colloids from 23·4 l. of urine were processed as described above and placed on the DEAE-cellulose column as in previous runs. The chromatogram obtained is shown in Fig. 5 and some analytical results for the various fractions are shown in Table 5. The chromatogram showed a distinctly different pattern from that seen in the normal urine or in pathological urine I, with much more material coming out in peak 1.

As in the previous two urines a high proportion of the non-dialysable calcium (32%) was associated with fractions 9, 10 and 11, which contained little ultraviolet-absorbing material.

Reactions against antisera to human-serum albumin and human-serum orosomucoid were positive for fractions 7 and 8 only. This urine contained a relatively larger amount of fraction 1 and a lower amount of fractions 7 and 8 than the previous two urines. As already mentioned, fractions 2–8 contained several antigens reacting with antibodies prepared to normal human serum.

Investigation of the urine-colloid fractions for calcium-binding ability

In an effort to determine whether the total calcium-binding capacity in patients deviated from normal and might be related to the formation of stones, the ability of various urine-colloid

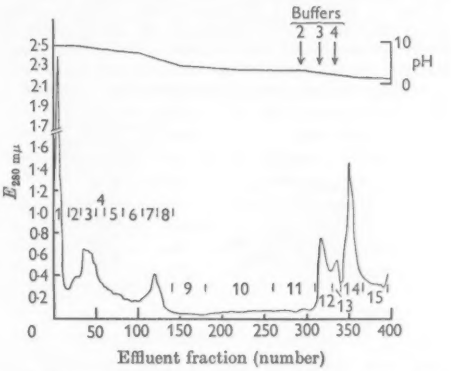

Fig. 5. Chromatogram of the colloids from a stone-free poliomyelitis urine obtained after fractionation on DEAE-cellulose.

Table 5. *Percentage composition of fractions from poliomyelitis urine II obtained by elution from DEAE-cellulose column*

Fraction no.	Yield (mg.)	Concn. in effluent (mg./ml.)	Hexuronic acid (%)	Calcium (%)
1	157·3	1·00	1·71	0
2	29·3	0·20		
3	52·5	0·24	—	0·38
4	12·8	0·11		
5	22·2	0·12	—	0·45
6	22·4	0·09	—	0·67
7	35·8	0·20	1·31	0·11
8	27·6	0·12	1·30	0·38
9	16·3	0·04	—	1·93
10	22·3	0·03	2·00	0·53
11	25·4	0·04	1·82	0·74
12	28·7	0·09	14·4	0·83
13	23·1	0·14	12·9	0·50
14	24·0	0·07	6·35	0·82
15	31·4	0·06		
Total:	531·1			

fractions to bind calcium was investigated. The colloids from 24 hr. urine specimens derived from normal subjects, and also from patients recovering from poliomyelitis (many of whom had formed stones), were used.

Table 6 illustrates some typical results obtained for several colloid fractions: $ChSO_4$ refers to the potassium salt of chondroitin sulphate isolated from nasal cartilage by the method of Einbinder & Schubert (1951); A_5 was a glycoprotein-rich fraction isolated from normal urine by the benzoic acid-adsorption method (Anderson & Maclagan, 1955); HSA refers to human-serum albumin; NUUC (normal) and (path.) refer to the non-ultrafiltrable colloids isolated by the method here described from the urine of normal subjects and from the urine of a patient with bilateral calculi.

Table 6. *Calcium-binding ability of colloids*

$ChSO_4$ is the potassium salt of chondroitin sulphate isolated from bovine nasal cartilage by the method of Einbinder & Schubert (1951). A_5 is a glycoprotein-rich fraction isolated from normal urine by the benzoic acid method of Anderson & Maclagan (1955). HSA is human-serum albumin. NUUC (normal) is the non-ultrafiltrable urine colloid isolated from normal urine by the present method. NUUC (path.) is the corresponding fraction isolated from the urine of a patient with bilateral calculi.

Colloid	Bound calcium (μequiv./mg. of colloid)
$ChSO_4$	1·14
A_5	0·26
HSA	0·20
NUUC (normal)	0·45
NUUC (path.)	0·56

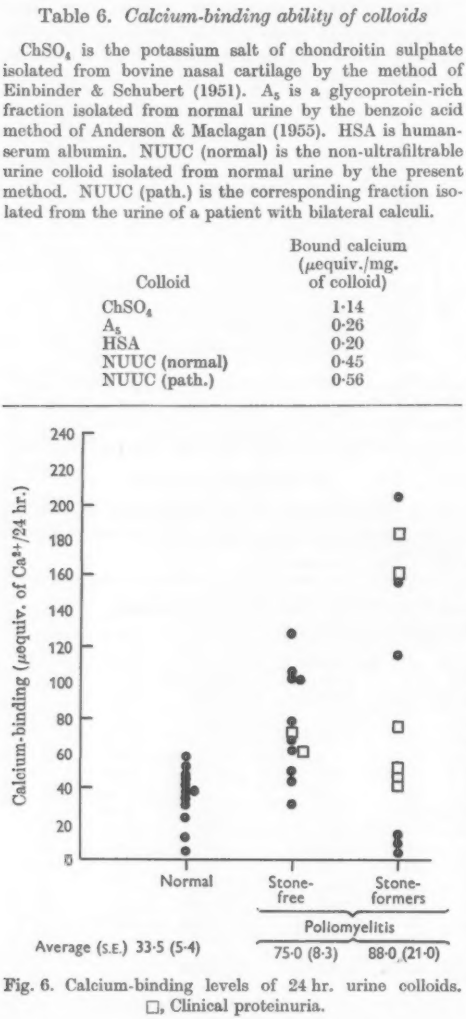

Fig. 6. Calcium-binding levels of 24 hr. urine colloids. □, Clinical proteinuria.

The calcium-binding abilities of the normal colloids obtained from the DEAE-cellulose column (Fig. 1) are shown in Table 3. Calcium-binding by the fractions from pathological urine I (Fig. 3) are contained in Table 4.

From these data no relationship is evident between the extent of calcium-binding and content of sialic acid, fucose, hexosamine or the non-dialysable calcium content of the chromatographic fractions from either urine sample. The normal colloid fractions 1–8 (Table 3) contained relatively low amounts of hexuronic acid and low calcium-binding abilities. Fractions 10–16, however, with higher contents of hexuronic acid, appeared to bind calcium somewhat more strongly. The fractions from the poliomyelitis urine (Table 4) showed less relationship between their hexuronic acid contents and their calcium-binding abilities. It is therefore clear that factors other than the content of uronic acid-containing mucopolysaccharides must be important in the binding of calcium.

The calcium-binding levels of the colloids in 24 hr. urine specimens from both normal subjects and patients recovering from poliomyelitis are shown in Fig. 6. No correlation with the duration of the disease or with the daily urine-output volume was evident.

DISCUSSION

The ultrafiltration method for isolating the colloids from normal male urine gave yields of about 200 mg./l. of urine. At pH 9·0 these colloids were soluble only to the extent of 140 mg./l. These values are in essential accord with partition noted by Boyce *et al.* (1958). About 65% of the weight of the non-ultrafiltrable fractions could be accounted for on the basis of their content of hexose, hexosamine, sialic acid, fucose, hexuronic acid and protein. The composition of these colloids, as has previously been demonstrated by Tamm & Horsfall (1952), Anderson & Maclagan (1955), Craddock & Kerby (1955), Hammerman & Hatch (1955), Baar (1956), DiFerrante & Rich (1956), Bonomo, Salteri & Cirla (1958), King *et al.* (1958, 1959), Boyce *et al.* (1958), Boyce & King (1959) and others, indicates the presence of relatively large amounts of glycoproteins and mucopolysaccharides.

The separation of the colloids by the DEAE-cellulose procedure of Sober & Peterson (1958), modified to have a steeper gradient of pH and salt concentration, has resulted in fractionation of the

urine colloids into several heterogeneous fractions of quite different composition. About 38% of the colloid added to the column was recovered in the various effluents.

The three chromatograms obtained by use of the tris–citrate buffer system that was devised (Table 2) can be divided into three distinct parts. Some components of the first eight fractions emerging appeared to contain aromatic amino acids, and on immunochemical evidence were in part derived from serum proteins. However, the percentage of hexose, hexosamine and sialic acid were considerably higher than values for the total serum proteins.

The presence of serum proteins in urine has been demonstrated immunoelectrophoretically by Grant (1957, 1959) and Patte, Baldassaire & Loret (1958). Two of these components are serum albumin and orosomucoid, which were detected immunochemically in the normal fractions 6–8. In the normal urine fractions, reactions with anti-orosomucoid serum were feeble, indicating a low concentration of urinary orosomucoid and that orosomucoid is not a major glycoprotein of normal urine, although it has been found to be relatively abundant in urines from patients with nephrosis (Popenoe, 1955). The urine-glycoprotein fraction prepared by adsorption on to benzoic acid (Anderson & Maclagan, 1955) is known to be heterogeneous, both on electrophoresis (Biserte, Tayeau, Montreuil, Holleman & Dautrevaux, 1956) and after fractionation on DEAE-cellulose (Kickhöfen *et al.* 1958). It is probable that some of the components of this fraction are of pre-renal origin. The electrophoretic properties at pH 8·6 of fractions 1–8 (Figs. 2 and 4) showed the general trend, observed with the serum proteins, toward higher mobilities in fractions coming off the column at lower pH. These fractions also stained strongly with protein-staining dyes.

The second section (fractions 9–11) contained little ultraviolet-absorbing material but considerable hexose, uronic acid, hexosamine and calcium. These fractions stained very feebly with protein-staining dyes, and were either immobile on electrophoresis at pH 8·6 and 4·6 or had mobilities mainly in the α- and β-globulin range.

The third group (fractions 12–16) absorbed at 280 mμ and contained in addition appreciable amounts of hexuronic acid. Fraction 12 (Fig. 1) had a considerable amount of negatively charged material so far not detected in serum, much of which migrated on paper electrophoresis at pH 4·6 considerably further than serum orosomucoid. Similar fast-migrating material had been noted previously by Markham, Jacobs & Fletcher (1956), who observed two very acidic electrophoretic components in urine, both of which stained more strongly for carbohydrate than for protein. These protein colloids with low isoelectric points may well be included in fraction 12.

The distribution of colloids capable of inhibiting haemagglutination of erythrocytes by virus into a number of widely separable components suggests either that the urinary glycoprotein of Tamm & Horsfall (1950) is easily depolymerized into active sub-units (Porter & Tamm, 1955) or that there are several inhibitory substances in urine. At least two viral haemagglutin inhibitors with differing electrophoretic mobilities have been demonstrated in serum (Burnet, 1951; Tyrrell, 1954), these being immunochemically distinct from the Tamm & Horsfall glycoprotein (Grant, 1957).

There is at present little information about the binding of calcium by urine-colloid fractions. Calcium-binding by various serum proteins, however, has been investigated by many workers. It is generally considered that albumin binds the most calcium (Schmidt & Greenberg, 1935; Prasad & Flink, 1958) and some binding would appear to be related to the presence in serum of proteins of phospholipid (Drinker & Zinsser, 1943).

In this investigation, the more acidic fractions isolated from normal urine (fractions 12–16, Table 3) were the strongest calcium-binders.

The urine colloids from poliomyelitis patients (Fig. 6) bound calcium more strongly than the colloids from a series of normal individuals, although there was no significant difference between the stone-free and stone-forming poliomyelitis urines.

It is unlikely that these poliomyelitis urines, except those exhibiting proteinuria, contain quantitatively more colloidal material than normal urines, since in previous studies (Maclagan & Anderson, 1958 *a*, *b*) no significant increase of the urine colloids above normal levels could be detected in a series of patients with renal calculi. It is more likely that a qualitative difference is present, there being an increased excretion of colloidal material in poliomyelitis with strong affinity for calcium.

This work has served to illustrate the wide distribution among the various urine fractions of considerable amounts of carbohydrate-containing colloids of which virtually nothing is known. The evidence presented here would suggest the presence of many glycoprotein entities, some of which, like orosomucoid, are derived from the blood, whereas others, like the glycoprotein isolated by Tamm & Horsfall (1950, 1952), may originate from the urinary tract. The source of the mucopolysaccharides is unknown.

The results presented here suggest that the proteins in urine can be separated on the anion-exchanger DEAE-cellulose. This method, however, does not appear as satisfactory for the separation

of mucopolysaccharides since it is probable that non-electrostatic forces are involved in their retention by the cellulose, most of the mucopolysaccharides being liberated only at very low pH values. Owing to the complexity and diversity of the colloids in urine it is unlikely that an ideal separation will be achieved by the use of any one method, and some combination of several techniques will be required in order to isolate colloids in a homogeneous state.

SUMMARY

1. An ultrafiltration method for recovering the colloids from normal human urine has been presented, and fractionation of these colloids has been carried out on the ion-exchanger DEAE-cellulose, a 2-amino-2-hydroxymethylpropane-1:3-diol–citrate buffer system being used as the eluent, with a gradually descending pH from 9·0 to 1·5. The colloids in each fraction have been isolated by freeze-drying and analysed for hexose, hexosamine, sialic acid, fucose, hexuronic acid, nitrogen and calcium. Their electrophoretic properties at pH 8·6 and 4·6 have also been reported.

2. The ability of these colloid fractions to inhibit the haemagglutination caused by influenza virus has been determined, as has their ability to react with antibodies produced against human-serum albumin and orosomucoid.

3. Fractionation of the urine colloids from two extensively paralysed patients with poliomyelitis, one of whom was forming renal calculi, revealed qualitatively similar chromatographic patterns with certain quantitative differences.

4. The ability of the colloid fractions to bind calcium has been measured by an equilibrium-dialysis technique. Calcium-binding by total non-dialysable urine colloids was found to be significantly above normal in the urine of patients recovering from poliomyelitis. No difference could be detected, however, between the urines from patients who were forming renal calculi and those who were not.

We are very grateful to Dr W. H. Boyce for samples of uromucoid and to Dr B. Weissmann for the preparation of chondroitin sulphate. We wish to thank Professor Otto Westphal and Otto Ludevitz for a generous gift of lipopolysaccharide isolated from *Escherichia coli*. This work was supported by a grant from the National Foundation.

REFERENCES

Anderson, A. J. (1954). *Biochem. J.* **56**, xxv.
Anderson, A. J., Lockey, E. & Maclagan, N. F. (1955). *Biochem. J.* **60**, xli.
Anderson, A. J. & Maclagan, N. F. (1955). *Biochem. J.* **59**, 638.
Baar, S. (1956). *J. clin. Path.* **9**, 144.
Biserte, G., Tayeau, F., Montreuil, J., Holleman, J. & Dautrevaux, N. (1956). *Clin. chim. Acta*, **1**, 115.
Björnesjö, K. B., Werner, I. & Odin, L. (1959). *Scand. J. clin. Lab. Invest.* **7**, 238.
Bonomo, E., Salteri, F. & Cirla, E. (1958). *Arch. Stud. Fisiopat.* **22**, 49.
Boyce, W. H., Garvey, F. K. & Norfleet, C. M. (1954). *J. clin. Invest.* **33**, 1287.
Boyce, W. H. & King, J. S. (1959). *J. clin. Invest.* **38**, 1525.
Boyce, W. H., King, J. S., Little, J. M. & Artom, C. (1958). *J. clin. Invest.* **38**, 1658.
Boyce, W. H. & Swanson, M. (1955). *J. clin. Invest.* **34**, 1581.
Burnet, F. M. (1951). *Physiol. Rev.* **31**, 131.
Craddock, J. C. & Kerby, G. P. (1955). *J. Lab. clin. Med.* **46**, 193.
DiFerrante, N. & Rich, C. (1956). *Clin. chim. Acta*, **1**, 519.
Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
Dische, Z. & Shettles, L. B. (1948). *J. biol. Chem.* **175**, 595.
Drinker, N. & Zinsser, H. H. (1943). *J. biol. Chem.* **148**, 187.
Durram, E. L. (1950). *J. Amer. chem. Soc.* **72**, 2943.
Einbinder, J. & Schubert, M. (1951). *J. biol. Chem.* **191**, 591.
Faillard, H. (1956). *Hoppe-Seyl. Z.* **305**, 145.
Ferro, P. & Hamm, A. B. (1957). *Amer. J. clin. Path.* **28**, 689.
Flynn, F. V. & De Mayo, P. (1951). *Lancet*, ii, 235.
Goodman, M., Ramsey, S. S., Simpson, W. L., Ranip, D. G., Basinski, D. H. & Brennan, M. J. (1957). *J. Lab. clin. Med.* **49**, 151.
Grant, G. H. (1957). *J. clin. Path.* **10**, 360.
Grant, G. H. (1959). *J. clin. Path.* **12**, 510.
Hammerman, D. & Hatch, F. T. (1955). *Proc. Soc. exp. Biol., N.Y.*, **89**, 279.
Heremans, J. (1958). *Clin. chim. Acta*, **3**, 34.
Jacques, L. B., Bapke, E. & Levy, S. W. (1953). *Circulation Res.* **1**, 321.
Kabat, E. A. & Meyer, M. M. (1948). *Experimental Immunochemistry*. Springfield, Ill.: Charles C. Thomas Publisher.
Kerby, G. P. (1954). *J. clin. Invest.* **33**, 1168.
Kickhöfen, B., Struwe, F. E., Bramesfeld, B. & Westphal, O. (1958). *Biochem. Z.* **330**, 467.
King, J. S., Boyce, W. H., Little, J. M. & Artom, C. (1958). *J. clin. Invest.* **37**, 315.
King, J. S., Little, J. M., Boyce, W. H. & Artom, C. (1959). *J. clin. Invest.* **38**, 1520.
Laurent, B. (1958). *Scand. J. clin. Lab. Invest.* **10**, Suppl. 32.
Lockey, E., Anderson, A. J. & Maclagan, N. F. (1956). *Brit. J. Cancer*, **10**, 209.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
McGarry, E., Schon, A. J. & Rose, B. (1955). *J. clin. Invest.* **34**, 832.
Maclagan, N. F. & Anderson, A. J. (1958*a*). *Brit. J. Urol.* **30**, 269.
Maclagan, N. F. & Anderson, A. J. (1958*b*). *Ciba Foundation Symp., The Chemistry and Biology of Mucopolysaccharides*, p. 268.
Markham, R. L., Jacobs, J. H. & Fletcher, E. T. D. (1956). *J. Lab. clin. Med.* **48**, 559.

Möerner, A. H. (1895). *Skand. Arch. Physiol.* **6**, 332.
Patte, J. C., Baldassaire, G. & Loret, J. (1958). *Rev. franç. Étud. clin. biol.* **3**, 960.
Peterson, H. A. & Sober, E. A. (1958). *Fed. Proc.* **17**, 288.
Popenoe, E. A. (1955). *J. biol. Chem.* **217**, 61.
Porter, K. R. & Tamm, I. (1955). *J. biol. Chem.* **212**, 135.
Prasad, A. S. & Flink, E. B. (1958). *J. Lab. clin. Med.* **51**, 345.
Rich, C. & DiFerrante, N. (1956). *Clin. Chem.* **2**, 235.
Rigas, D. A. & Heller, C. G. (1951). *J. clin. Invest.* **30**, 853.
Rondle, C. J. M. & Morgan, W. T. J. (1955). *Biochem. J.* **61**, 586.
Schmidt, C. L. A. & Greenberg, D. N. (1935). *Physiol. Rev.* **15**, 297.
Shatton, J. & Schubert, M. (1954). *J. biol. Chem.* **211**, 565.
Sober, H. A., Cutter, F. J., Wyckoff, M. M. & Peterson, E. A. (1956). *J. Amer. chem. Soc.* **78**, 756.
Sober, H. A. & Peterson, E. A. (1958). *Fed. Proc.* **17**, 1116.
Sonnet, J. (1956). *Rev. belge. Path.* **25**, 498.
Tamm, I., Bugher, J. C. & Horsfall, F. L. (1955). *J. biol. Chem.* **212**, 125.
Tamm, I. & Horsfall, F. L. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 108.
Tamm, I. & Horsfall, F. L. (1952). *J. exp. Med.* **95**, 71.
Tyrrell, D. A. J. (1954). *J. Immunol.* **72**, 494.
Werner, I. & Odin, L. (1952). *Acta Soc. Med. Upsalien.* **57**, 230.
Williams, C. A. & Grabar, P. (1955). *J. Immunol.* **74**, 158.
Winzler, R. J. (1955). *Meth. biochem. Anal.* **2**, 279.

Biochem. J. (1960) **77**, 591

The Mode of Recovery of Cholinesterase Activity *in vivo* after Organophosphorus Poisoning

1. ERYTHROCYTE CHOLINESTERASE

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It is now generally accepted that the inhibition of cholinesterase by organophosphorus compounds consists of a direct phosphorylation of the active centre with the formation of a disubstituted phosphoryl cholinesterase (Aldridge, 1953). If the inhibited enzyme is kept *in vitro* in the absence of excess of inhibitor, a certain amount of spontaneous reactivation occurs by hydrolysis of the phosphorylated enzyme, the exact amount being dependent on the groups attached to the phosphorus atom (Aldridge, 1953; Burgen & Hobbiger, 1951; Aldridge & Davison, 1953).

Reactivation of the inhibited cholinesterase can be markedly accelerated by treating it with certain nucleophilic reagents such as choline (Wilson, 1951), hydroxamic acids (Wilson, 1955) and oximes (Childs, Davies, Green & Rutland, 1955; Wilson & Ginsberg, 1955; Davies & Willey, 1957), the rate and extent of reactivation again being dependent on the groups attached to the phosphorus atom. The oximes are by far the best reactivators and under suitable conditions, with freshly inhibited enzyme, oxime treatment will restore all the enzyme activity (Davies & Green, 1956).

If the freshly inhibited and completely reactivatable cholinesterase is stored it gradually changes into a form which can no longer be reactivated by oximes (Davies & Green, 1956; Wilson, Ginsberg & Meislich, 1955; Hobbiger, 1955; Hobbiger, 1956; Jandorf, Michel, Schaffer, Egan & Summerson, 1955). This may be due either to the migration of the phosphoryl group to a more stable position in the inhibited enzyme (Wilson, Ginsberg & Meislich, 1955; Hobbiger, 1956; Jandorf *et al.* 1955), or to loss of one of the substituents on the phosphorus atom which would then acquire a negative charge and consequently become resistant to nucleophilic attack (Oosterbaan, Warringa, Jansz, Berends & Cohen, 1958). The change which occurs during storage will be referred to here as 'aging' although some authors have in fact referred to it as a transphosphorylation process (Hobbiger, 1956; Jandorf *et al.* 1955).

Little is known about the extent to which these reactions influence the *in vivo* recovery of inhibited cholinesterase. Davison (1955) suggested that the return to normal levels of activity was probably the combined result of spontaneous hydrolysis of the inhibited enzyme and of new enzyme synthesis. He found that after inhibition *in vivo* by diethyl *p*-nitrophenyl phosphate (E 600) the activities of rat-brain cholinesterase and of a variety of erythrocyte cholinesterases returned to normal in two

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stages, a rapid partial recovery followed by a slower completion of the recovery process. Davison (1955) attributed this to the existence of two types of cholinesterase which formed inhibited enzymes of different stabilities.

The object of the present work was to ascertain whether the recovery of erythrocyte cholinesterase activity *in vivo* after inhibition by various organophosphorus compounds could be accounted for in terms of spontaneous reactivation, aging and the synthesis of new enzyme. The inhibitors used were chosen so that inhibited cholinesterases of widely differing stabilities would be produced.

MATERIALS AND METHODS

Inhibitors. These were synthesized in the Chemistry Section, Chemical Defence Experimental Establishment (C.D.E.E.), by the following methods: di*iso*propyl phosphorofluoridate (DFP), Ford-Moore, Lermit & Stratford (1953); tetraethyl pyrophosphate (TEPP), Arbusow & Arbusow (1932); *iso*propyl methylphosphonofluoridate (Sarin), Bryant, Ford-Moore, Perry, Wardrop & Watkins (1960); dimethyl *p*-nitrophenyl phosphate (DMNP) was presented by Dr W. N. Aldridge, Toxicology Research Unit, Carshalton, Surrey.

Reactivators. These were also synthesized in the Chemistry Section, C.D.E.E.: mono*iso*nitrosoacetone (hydroxyiminoacetone; MINA) by the method of Freon (1939) and 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate (P2S) by the method of Creasey & Green (1959).

Special chemicals. Acetylcholine chloride was supplied by Roche Products Ltd.

Animals. Sheep were adult females or castrated males 40–60 kg.; rabbits were albino males of approx. 2 kg.

Preparation of 'packed erythrocytes'. The plasma was removed from a sample of heparinized whole blood by centrifuging. The erythrocytes were then washed twice with 10 times their own volume of 0·9% NaCl soln. and packed by centrifuging at 1500 *g* for 10 min. The NaCl soln. was removed by aspiration and the cells were thoroughly stirred before sampling.

Estimation of cholinesterase activity. (*a*) Cholinesterase was estimated manometrically at 37° with the following reaction mixture: 0·1 ml. of 'packed erythrocytes' or 0·2 ml. of 50% erythrocyte suspension, 0·5 ml. of 0·12 M-$NaHCO_3$, 0·5 ml. of 0·036 M-acetylcholine chloride, water to 3·0 ml.; gas phase $N_2 + CO_2$ (95:5). Activity was expressed as μl. of CO_2/30 min./0·1 ml. of 'packed erythrocytes', allowance being made for non-enzymic hydrolysis.

(*b*) In certain experiments cholinesterase activity was estimated by continuous titration; 0·2 ml. of 'packed erythrocytes' was haemolysed in 2·0 ml. of water and 5·0 ml. of 0·007 M-acetylcholine chloride–0·42 M-KCl added. After equilibration at 25° the pH was adjusted to 7·5 and the mixture titrated continuously by the addition of portions (2 μl.) of 0·1 N-NaOH over a period of 6 min. The time was noted at which each addition of NaOH was neutralized and a titration curve was plotted. The slope was measured and activity expressed as μl. of 0·1 N-NaOH used/0·1 ml. of 'packed erythrocytes'/min.

Rate of reactivation of inhibited erythrocyte cholinesterase by 2-(hydroxyiminomethyl)-N-methylpyridinium methanesulphonate and monoisonitrosoacetone. 'Packed erythrocytes', diluted with an equal volume of 0·9% NaCl, were mixed with equal volumes of 2 μM-DMNP, 2 μM-TEPP, 2 μM-Sarin or 20 μM-DFP, all solutions were in 0·9% NaCl, and the mixtures were kept at 37° for 30 min. Excess of inhibitor was then removed by washing the erythrocytes at least three times with 10 times their volume of 0·9% NaCl soln. Samples of the inhibited erythrocytes were then mixed with an equal volume of 2 mM-2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate in 0·9% NaCl or with an equal volume of 20 mM-mono*iso*nitrosoacetone in 0·9% NaCl and kept at room temperature. Samples of the mixtures were removed at intervals, the erythrocytes were washed twice with 10 times their volume of 0·9% NaCl to remove excess of oxime and finally packed by centrifuging at 1500 *g* for 10 min. After removing the supernatant NaCl soln., the erythrocyte cholinesterase activity was measured manometrically.

Measurement of aging. With cholinesterase inhibited by DMNP, TEPP or DFP equal volumes of 'packed erythrocytes' and 2 mM-2-(hydroxyiminomethyl)-*N*-methylpyridinium methane sulphonate were mixed and kept at room temperature for 1 hr. at pH 7·5. With Sarin-inhibited cholinesterase the erythrocytes were mixed with an equal volume of 20 mM-MINA and kept for 1 hr. The erythrocytes were then washed twice with 10 times their volume of 0·9% NaCl soln. to remove excess of oxime and packed by centrifuging at 1500 *g* for 10 min. After removing the supernatant the cholinesterase activity of the erythrocytes was measured manometrically.

The percentage of aged enzyme was that percentage of the initial activity which could not be recovered by treatment with oximes.

Inhibition in vivo. Sheep were given 0·045 mg. of atropine sulphate/kg. subcutaneously; 10 min. later, solutions of the inhibitors in 0·9% NaCl soln. were injected as follows: TEPP (0·1 mg./kg.) and Sarin (0·015 mg./kg.) were given subcutaneously; DFP (0·5 mg./kg.) or DMNP (0·2 mg./kg.) was given intravenously, DFP because subcutaneous administration produced only a slow inhibition, and DMNP because the inhibited cholinesterase has a half-life of only 90 min. (Aldridge & Davison, 1953) and it was therefore necessary to bring the enzyme and inhibitor into contact as quickly as possible. Subcutaneous injections were given in the axilla and intravenous injections into the jugular vein.

Storage and recovery of inhibited erythrocyte cholinesterase in vivo and in vitro. At suitable intervals after injecting the inhibitors (5 min. after DMNP, 30 min. after TEPP and Sarin, and 4 hr. after DFP) 10–20 ml. of blood was withdrawn, 'packed erythrocytes' were prepared and the cholinesterase activity was determined manometrically before and after oxime treatment as described under 'measurement of aging'.

The packed erythrocytes remaining after the cholinesterase determination were mixed with an equal volume of the following preservative solution: potassium oxalate 1·8%, 8-hydroxyquinoline 0·1%, NaCl 0·9%, glucose 6·48%, and stored at pH 7·4 and 37°. When stored in this manner normal erythrocytes retained their cholinesterase activity for 7 days, very little haemolysis occurred and bacterial growth was prevented. Samples were taken at the same time from both the stored erythrocytes and from the

Table 1. *Reactivation of inhibited sheep-erythrocyte cholinesterase by oximes*

The inhibited erythrocyte cholinesterase was incubated at 37° with the stated concentration of oxime. At the times stated samples were taken, the erythrocytes washed with 0·9% NaCl and the cholinesterase activity was measured. The initial inhibition of the cholinesterase *in vitro* was produced as described under Methods.

Time of contact with oxime (min.)	Percentage of initial cholinesterase activity							
	DMNP		TEPP		DFP		Sarin	
	10^{-3}M P2S	10^{-2}M MINA	10^{-3}M P2S	10^{-2}M MINA	10^{-3}M P2S	10^{-2}M MINA	10^{-3}M P2S	10^{-2}M MINA
0	37·0	37·0	18·0	18·0	43·0	43·0	15·0	15·0
30	—	50·5	100·0	51·5	49·0	47·0	71·0	79·5
45	84·0	—	—	—	—	—	—	—
60	—	—	100·0	63·5	55·5	53·0	81·0	100·0
90	84·0	—	—	—	—	—	—	—
120	—	85·5	100·0	89·5	71·0	60·5	100·0	100·0
180	87·5	—	100·0	88·0	67·5	56·0	100·0	100·0
240	—	85·5	100·0	95·5	75·0	60·5	100·0	100·0
300	85·5	—	—	—	—	—	—	—
360	—	—	—	—	77·5	72·5	—	—

sheep, and the erythrocyte cholinesterase activity was estimated before and after treatment with oxime. All the erythrocyte cholinesterase activities were expressed as a per cent of a normal value determined immediately before the sheep was poisoned.

RESULTS

Measurement of the 'aging process'

In order to follow the aging process one must be able to reactivate completely the non-aged inhibited cholinesterase present at any particular time. Ideally, therefore, the rate of reactivation by oxime should be very much greater than the rate of the aging process. With TEPP- and Sarin-inhibited cholinesterase, treatment with the appropriate oxime for 1 hr. resulted in complete reactivation of the inhibited enzyme, consequently the aging process could easily be followed. Table 1 shows that for TEPP-inhibited cholinesterase 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate was the faster reactivator and for Sarin-inhibited cholinesterase MINA was the faster reactivator; these combinations of inhibited cholinesterase and oxime were therefore used in the routine aging measurements. With DFP- and DMNP-inhibited cholinesterase, however, complete reactivation of the inhibited enzyme could not be observed. Reactivation of the DFP-inhibited cholinesterase continued for 4–5 hr. and became very slow when only about 50% of the inhibited enzyme initially formed was reactivated. Reactivation of the DMNP-inhibited cholinesterase ceased after contact for 45 min. with the oxime with about 76% of the inhibited enzyme initially formed being reactivated. It is clear that the reactivation and aging of DFP- and DMNP-inhibited cholinesterase proceeded at comparable rates, and although it was possible to tell whether the aging process was still proceeding, it was not possible to observe it as accurately as with Sarin- and TEPP-inhibited cholinesterase.

Recovery of erythrocyte cholinesterase activity in poisoned sheep

Preliminary experiments with rabbits showed that small animals could not be used in this work because the removal of the necessary quantities of blood, in all a considerable proportion of the total blood volume, caused an increase in the proportion of circulating reticulocytes, blood constituents which contain three times the cholinesterase activity associated with a normal red-cell population (Pritchard, 1949; Allison & Burn, 1955). The erythrocyte cholinesterase activity of the poisoned rabbits consequently returned much faster than could be accounted for either by published rates of erythrocyte turnover (Neuberger & Niven, 1951), or by the rate of spontaneous reactivation of the inhibited cholinesterase as observed *in vitro*. No such difficulty was experienced with sheep.

To see whether spontaneous reactivation, aging and new enzyme synthesis could satisfactorily explain the changes in the erythrocyte cholinesterase activity during the recovery from poisoning, a sample of blood was taken from a sheep soon after it had received a non-lethal dose of the cholinesterase inhibitor. The erythrocytes were separated, stored at 37° with a preservative and the rates of spontaneous reactivation and aging in the stored erythrocytes compared with their rates in the circulating erythrocytes of the intact sheep. Each inhibitor was given to three sheep and typical results are shown in Figs. 1–4.

The return of inhibited erythrocyte cholinesterase to the normal level of activity *in vivo* occurred

in one or two stages depending on which inhibitor was used.

With TEPP-, Sarin- or DMNP-poisoned sheep the recovery of inhibited erythrocyte cholinesterase *in vivo* occurred in two stages, a rapid partial recovery being followed by a slower linear return to the normal level of activity. *In vitro* only the first stage was observed.

Figs. 1–3 show that the rates of the separate reactions involved in the first stage, namely spontaneous reactivation and aging were the same *in vivo* as they were *in vitro*. Both reactions occurred simultaneously and when all the residual inhibited enzyme was converted into the aged form no further spontaneous reactivation appeared

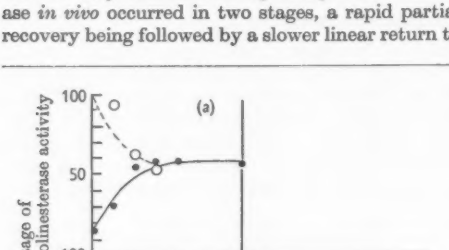

Fig. 1. Behaviour *in vitro* (*a*) and *in vivo* (*b*) of erythrocyte cholinesterase of sheep poisoned with 0·1 mg. of TEPP/kg. subcutaneously. Broken lines show the decrease in cholinesterase reactivatable by P2S and represent the aging of the inhibited enzyme. Full lines show the cholinesterase activity before oxime treatment and represent spontaneous recovery (*in vitro*) or spontaneous recovery plus new enzyme synthesis (*in vivo*).

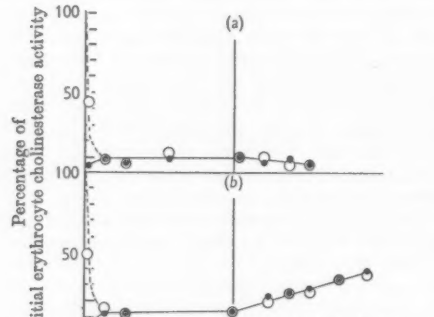

Fig. 2. Behaviour *in vitro* (*a*) and *in vivo* (*b*) of erythrocyte cholinesterase of sheep poisoned with 0·015 mg. of Sarin/kg. subcutaneously. Broken lines show the decrease in cholinesterase reactivatable by mono*iso*nitrosoacetone and represent the aging of the inhibited enzyme; full lines show the cholinesterase activity before oxime treatment and represent spontaneous recovery (*in vitro*) or spontaneous recovery plus new enzyme synthesis (*in vivo*).

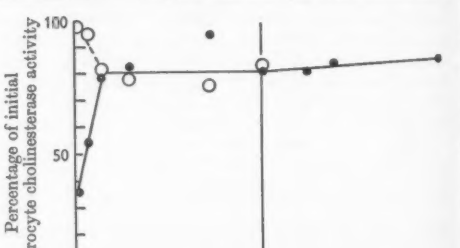

Fig. 3. Behaviour *in vivo* of erythrocyte cholinesterase of sheep poisoned with 0·2 mg. of DMNP/kg. intravenously; the broken line shows the decrease in cholinesterase reactivatable by P2S and represents the aging of the inhibited enzyme; the full line shows the cholinesterase activity before oxime treatment and represents spontaneous recovery plus new enzyme synthesis.

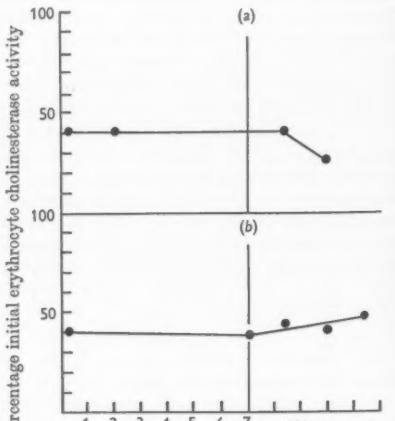

Fig. 4. Behaviour of erythrocyte cholinesterase of sheep poisoned with 0·5 mg. of DFP/kg. intravenously. Spontaneous reactivation did not occur *in vitro* (*a*) and the increase in erythrocyte cholinesterase activity *in vivo* (*b*) was due solely to new enzyme synthesis.

to be possible. The relative rates of the two processes determined the extent to which the inhibited enzyme recovered spontaneously. For example, in the case of the TEPP-inhibited enzyme spontaneous reactivation proceeded at the same rate as aging and at the end of the first stage 49% of the inhibited activity had recovered *in vitro*; with the Sarin-inhibited enzyme, on the other hand, the aging process was much faster than spontaneous reactivation and at the end of the first stage only 2% of the inhibited activity had returned *in vitro*. The absolute rates of the two reactions determined how long the first stage of the recovery lasted; thus with Sarin the first stage was completed in 24 hr., whereas with TEPP the first stage lasted 3 days.

Spontaneous reactivation and aging were observed *in vivo* with DMNP-inhibited cholinesterase (Fig. 3), although the rates were much faster than with either Sarin- or TEPP-inhibited cholinesterase, the first stage being completed in 1 hr. Because of the difficulty of completing all the analyses on the same animal during the first hour, only the behaviour of DMNP-inhibited cholinesterase *in vivo* was studied. However, the rate of spontaneous reactivation *in vitro* has been studied by Aldridge & Davison (1953), who found that with rabbit erythrocytes the inhibited enzyme had a half-life of only 90 min.

With DFP-poisoned sheep (Fig. 4) no spontaneous reactivation was detected *in vitro*, and *in vivo* the return of the erythrocyte cholinesterase activity proceeded throughout the entire recovery period at the slower linear rate typical of the second stage. Aging of the DFP-inhibited enzyme appeared to be complete in about 6 hr. This could only be roughly assessed, however, because of the slow rate at which the inhibited enzyme was reactivated by 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate.

The rate of the later linear stage was similar for all the inhibitors used; thus with TEPP, Sarin, DFP and DMNP the daily rates at which the inhibited erythrocyte cholinesterase was replaced were 0·6, 0·7, 0·5 and 0·7% respectively, suggesting that the mechanism involved in this stage of the recovery was the same for each. As this rate of replacement is similar to that measured by different methods (Neuberger & Niven, 1951) for the normal turnover of erythrocytes in other mammals the second or linear stage in the recovery of inhibited erythrocyte cholinesterase activity was assumed to be due to new red-cell formation.

DISCUSSION

In the present experiments erythrocyte cholinesterase, inhibited with organophosphorus compounds, recovered in one or two stages depending on which inhibitor was used. During the first stage, which occurred both *in vivo* and *in vitro*, the inhibited enzyme underwent two simultaneous reactions: spontaneous reactivation by hydrolysis, and aging in which the inhibited enzyme was converted into a form which could no longer reactivate spontaneously or be reactivated by oxime treatment.

When all the inhibited enzyme was present in the aged form no further recovery of the erythrocyte cholinesterase activity occurred *in vitro*, and *in vivo* the second stage of recovery was reached in which the rate of erythrocyte cholinesterase recovery became that at which the inhibited erythrocytes were replaced by new ones. Thus the characteristics of the first stage were dependent on the inhibitor, whereas those of the second stage were not, and were the same for all the inhibitors used.

When the cholinesterase was inhibited with TEPP, Sarin or DMNP the two stages in the recovery could clearly be seen; with DFP-inhibited erythrocyte cholinesterase, however, no spontaneous recovery was detected *in vitro*, and *in vivo* only one stage of recovery was observed, the rate being that of new erythrocyte formation.

If failure to detect spontaneous reactivation was due to aging of the DFP-inhibited enzyme within the first hour or so, then it would not be possible to reactivate it by prolonged oxime treatment as has been described. The absence of spontaneous reactivation immediately after inhibition was therefore attributed to the freshly inhibited enzyme's being highly resistant to hydrolysis. It will be observed that the rate of spontaneous reactivation of the inhibited erythrocyte cholinesterase decreased markedly in the order dimethyl-, diethyl-, di*iso*propyl-phosphoryl cholinesterase.

The inhibition of cholinesterase and the first stage in its recovery may be summarized as shown in Fig. 5.

Since the nature of the inhibited enzyme produced by an organophosphorus compound depends on the dialkylphosphoryl residue, provided that this group is kept constant the same inhibited enzyme can be obtained from a variety of inhibitors differing from one another only in the 'X' group (Aldridge & Davison, 1953). This group may, however, modify the behaviour of the inhibitor *in vivo*. For example Vandekar & Heath (1957) found that, in contrast with DMNP, the dimethyl phosphate esters $(MeO)_2PO{\cdot}S{\cdot}C_2H_4R$, where R is –SEt, –SOEt or $-SO_2Et$, persisted in the tissues *in vivo*; and although spontaneous reactivation and aging of the inhibited cholinesterase proceeded normally, any free cholinesterase so produced was immediately reinhibited, so that eventually all the inhibited cholinesterase was present in the aged

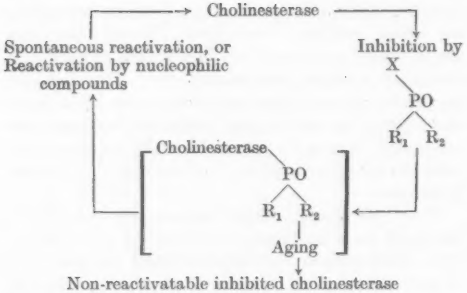

Fig. 5. Reactions occurring during and after the inhibition of cholinesterase by organophosphorus compounds. DFP: $R_1 = R_2 =$ *iso*propoxy, X = F; TEPP: $R_1 = R_2 =$ ethoxy, X = $-O \cdot PO(OEt)_2$; Sarin: R_1 = methyl, $R_2 =$ *iso*propoxy, X = F; DMNP: $R_1 = R_2$ = methoxy, X = *p*-nitrophenoxy.

form and no spontaneous recovery could be detected. Recovery of erythrocyte cholinesterase activity *in vivo* therefore occurred only at the slow linear rate associated with new erythrocyte formation.

Thus it is possible to explain the behaviour of erythrocyte cholinesterase inhibited *in vivo* by organophosphorus compounds in terms of the spontaneous hydrolysis and aging of the inhibited enzyme and the formation of new red cells.

The two forms of inhibited cholinesterase with different stabilities originally postulated by Davison (1955) are probably the freshly inhibited enzyme, which can undergo spontaneous reactivation, and the aged enzyme, which cannot. As the first form may undergo spontaneous and complete conversion into the second form there is no reason to believe that there are initially two types of uninhibited cholinesterase.

SUMMARY

1. Recovery of sheep-erythrocyte cholinesterase inhibited *in vivo* by dimethyl *p*-nitrophenyl phosphate, tetraethyl pyrophosphate or *iso*propyl methylphosphonofluoridate occurred in two stages, a rapid partial recovery being followed by a slower linear return to normal activity. With di*iso*propyl phosphorofluoridate-inhibited erythrocyte cholinesterase, recovery occurred throughout at the slower linear rate.

2. During the first stage in the recovery spontaneous hydrolysis and aging of the inhibited cholinesterase occurred simultaneously; the absolute rates of these two processes determined the speed of recovery and their relative rates determined the extent of recovery. The characteristics of this stage varied with the inhibitor used.

3. The second stage in the recovery was correlated with new red-cell formation and the rate of recovery in this stage, 0·5–0·7% per day, was independent of the inhibitor used.

REFERENCES

Aldridge, W. N. (1953). *Biochem. J.* **54**, 442.
Aldridge, W. N. & Davison, A. N. (1953). *Biochem. J.* **55**, 763.
Allison, A. C. & Burn, G. P. (1955). *Brit. J. Haematol.* **1**, 291.
Arbusow, A. E. & Arbusow, B. A. (1932). *Ber. dtsch. chem. Ges.* **65**, 195.
Bryant, P. J. R., Ford-Moore, A. H., Perry, B. J., Wardrop, A. W. H. & Watkins, T. F. (1960). *J. chem. Soc.* p. 1553.
Burgen, A. S. V. & Hobbiger, F. (1951). *Brit. J. Pharmacol.* **6**, 593.
Childs, A. F., Davies, D. R., Green, A. L. & Rutland, J. P. (1955). *Brit. J. Pharmacol.* **10**, 462.
Creasey, N. H. & Green, A. L. (1959). *J. Pharm., Lond.*, **11**, 485.
Davies, D. R. & Green, A. L. (1956). *Biochem. J.* **63**, 529.
Davies, D. R. & Willey, G. L. (1957). *Brit. J. Pharmacol.* **13**, 202.
Davison, A. N. (1955). *Biochem. J.* **60**, 339.
Ford-Moore, A. H., Lermit, L. J. & Stratford, C. (1953). *J. chem. Soc.* p. 1776.
Freon, P. (1939). *Ann. Chim.* **11**, 460.
Hobbiger, F. (1955). *Brit. J. Pharmacol.* **10**, 356.
Hobbiger, F. (1956). *Brit. J. Pharmacol.* **11**, 295.
Jandorf, B. J., Michel, H. O., Schaffer, N. K., Egan, R. & Summerson, W. H. (1955). *Disc. Faraday Soc.* **20**, 134.
Neuberger, A. & Niven, J. A. (1951). *J. Physiol.* **112**, 292.
Oosterbaan, R. A., Warringa, M. G. P. J., Jansz, H. S., Berends, F. & Cohen, J. A. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, no. 4–12, p. 38.
Pritchard, J. A. (1949). *Amer. J. Physiol.* **158**, 72.
Vandekar, M. & Heath, D. F. (1957). *Biochem. J.* **67**, 202.
Wilson, I. B. (1951). *J. biol. Chem.* **190**, 111.
Wilson, I. B. (1955). *J. Amer. chem. Soc.* **77**, 2383.
Wilson, I. B. & Ginsberg, S. (1955). *Biochem. biophys. Acta*, **18**, 168.
Wilson, I. B., Ginsberg, S. & Meislich, E. R. (1955). *J. Amer. chem. Soc.* **77**, 4286.

The Mode of Recovery of Cholinesterase Activity *in vivo* after Organophosphorus Poisoning

2. BRAIN CHOLINESTERASE

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Previous investigators (Frawley, Hagan & Fitzhugh, 1952; Davison, 1955; Kewitz & Nachmansohn, 1957) have observed that the rate of recovery *in vivo* of inhibited brain true cholinesterase depended on which inhibitor was used. With di*iso*propyl phosphorofluoridate (Frawley *et al.* 1952; Davison, 1955; Kewitz & Nachmansohn, 1957) recovery was slow, whereas with tetraethyl pyrophosphate (Davison, 1955) and diethyl *p*-nitrophenyl phosphate (Davison, 1955; Kewitz & Nachmansohn, 1957) recovery was considerably more rapid and occurred in two stages, the first being faster than the second. Davison (1955) suggested that the two-stage recovery could be explained by the existence of two kinds of inhibited cholinesterase with differing stabilities.

In the previous paper (Blaber & Creasey, 1960) it was shown that the changes *in vivo* in sheep-erythrocyte cholinesterase activity after inhibition by a variety of organophosphorus compounds could be explained in terms of (i) spontaneous reactivation of the inhibited enzyme by hydrolysis, (ii) aging, i.e. the gradual conversion of the inhibited enzyme from a form which can be reactivated by oximes into one which cannot, and (iii) new enzyme synthesis.

In the present paper a similar analysis has been made of the behaviour of inhibited rat-brain cholinesterase; this is a more complicated system since both true cholinesterase and pseudocholinesterase are present and cannot easily be separated. The combined effects of spontaneous reactivation, aging, and new enzyme synthesis on the recovery of inhibited brain cholinesterase have been investigated by comparing the behaviour *in vivo* and *in vitro* of the inhibited enzyme. The inhibitors were those used previously in the study of inhibited erythrocyte cholinesterase activity (Blaber & Creasey, 1960), namely dimethyl *p*-nitrophenyl phosphate, di*iso*propyl phosphorofluoridate, tetraethyl pyrophosphate and *iso*propyl methylphosphonofluoridate. Although aging of inhibited brain cholinesterase *in vivo* is known to occur (Hobbiger, 1957), it has not previously been investigated in relation to the recovery *in vivo* of inhibited cholinesterase in the brain.

Whilst this work was in progress suspicion was cast upon conventional methods of cholinesterase assay by Kewitz & Nachmansohn (1957), who claimed that, unless extracellular inhibitor was removed from poisoned brains before they were homogenized, falsely low cholinesterase levels would be obtained. The results of the present investigation do not support the idea that the inhibitors used are held at extracellular sites. The cholinesterase activities, which are reported here and which were measured by conventional methods of assay, are therefore considered to be reliable.

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MATERIALS AND METHODS

Inhibitors. Di*iso*propyl phosphorofluoridate (DFP), tetraethyl pyrophosphate (TEPP), and *iso*propyl methylphosphonofluoridate (Sarin) were prepared in the Chemistry Section, C.D.E.E., by methods described by Blaber & Creasey (1960); dimethyl *p*-nitrophenyl phosphate (DMNP) was presented by Dr W. N. Aldridge, *N*-(2-diethylamino-*n*-propyl)phenothiazine hydrochloride (ethopropazine) was presented by May and Baker Ltd., and diethyl *p*-nitrophenyl phosphate (E 600) was obtained from Albright and Wilson Ltd.

Substrates. Acetylcholine chloride was supplied by Roche Products Ltd., and acetyl-β-methylcholine chloride by Savory and Moore Ltd.; butyrylcholine iodide was prepared in the Chemistry Section, C.D.E.E.

Preservatives. 8-Hydroxyquinoline was obtained from British Drug Houses Ltd., Merthiolate from Eli Lilly and Co. Ltd., and Nipasept (a mixture of the methyl, ethyl and propyl esters of *p*-hydroxybenzoic acid) from Nipa Laboratories Ltd.

Reactivators. Mono*iso*nitrosoacetone (MINA) and 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate (P2S) were prepared in the Chemistry Section, C.D.E.E., as described by Blaber & Creasey (1960).

Animals. Rats were male albino 200–250 g.; mice were male albino 18–22 g.

Preparation of 'brain homogenate'. Brains were carefully removed, washed with distilled water to remove any adhering blood and then blotted dry. The brains were weighed and homogenized in an all-glass homogenizer with

0·0312 M-$NaHCO_3$–0·15 M-NaCl, pH 7·8 (Davison, 1953), the final volume being adjusted so that 1·0 ml. was equivalent to 50 mg. of fresh brain.

Cholinesterase activity. This was estimated manometrically with the following reaction mixture: 0·5 ml. of 'brain homogenate', 0·5 ml. of 0·0312 M-$NaHCO_3$–0·15 M-NaCl pH 7·8, 0·5 ml. of 0·0828 M-acetylcholine chloride (Davison, 1953), 1·5 ml. of 0·1 mM-ethopropazine hydrochloride, freshly prepared in 0·0312 M-$NaHCO_3$–0·15 M-NaCl, pH 7·8, gas phase $N_2 + CO_2$ (95:5). Activity was expressed as μl. of CO_2/30 min./25 mg. of fresh brain, allowance being made for non-enzymic hydrolysis.

Inhibition in vivo. Rats were given 50 mg. of atropine sulphate/kg. subcutaneously; 10 min. later solutions of inhibitors in 0·9% NaCl were injected as follows: DMNP 2·0 mg./kg. intraperitoneally, TEPP 0·25 mg./kg. subcutaneously, DFP 3·0 mg./kg. subcutaneously, and Sarin 0·012 mg./kg. subcutaneously.

Reactivation of inhibited brain cholinesterase and measurement of aging. 1·0 ml. of 0·1 M-2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate was mixed with 100 ml. of Sarin-, DMNP-, or TEPP-inhibited 'brain homogenate' and kept at room temperature for 1 hr. The DFP-inhibited 'brain homogenate', because of its low rate of reactivation, was kept overnight with this concentration of 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate. During the subsequent cholinesterase assay these mixtures were sufficiently diluted to prevent the occurrence of any cholinesterase inhibition by the oxime. The 1% error in the cholinesterase estimations, caused by the addition of oxime solution, was neglected. The percentage of the initial activity which could not be recovered by treatment with oxime was the percentage of aged enzyme.

Storage of half-brains. Half-brains were stored at 37° in stoppered tubes with 0·1 ml. of a 0·05% solution of 8-hydroxyquinoline in 0·9% NaCl soln.

RESULTS

Preliminary investigations

Before the behaviour of inhibited brain true cholinesterase was studied *in vivo* and *in vitro* the following preliminary investigations were performed.

Choice of method for measuring the true cholinesterase activity of rat brain. The cholinesterase activity of rat brain when measured against acetylcholine consists of approximately 90% of the true enzyme and 10% of the pseudo enzyme (Davison, 1953). The true cholinesterase activity was therefore measured in the presence of a specific inhibitor for the small proportion of pseudocholinesterase. *NN'*-Di*iso*propylphosphorodiamidic fluoride (mipafox) (Davison, 1953), *NN'*-di*iso*propyl phosphorodiamidic anhydride (DPDA) (Austin & Berry, 1953), and *N*-(2-diethylamino-*n*-propyl)phenothiazine hydrochloride (ethopropazine) (Bayliss & Todrick, 1956) have previously been used for this purpose. In the present experiments ethopropazine was used (i) because of its higher specificity and (ii) because it is a reversible inhibitor and the degree of inhibition would not, therefore, depend on the time of contact.

In agreement with the findings of Bayliss & Todrick (1956) 50 μM-ethopropazine almost completely suppressed the hydrolysis of butyrylcholine by rat-brain homogenate but inhibited only 7% of the acetyl-β-methylcholine hydrolysis (Table 1). Because acetylcholine is hydrolysed faster than acetyl-β-methylcholine by true cholinesterase, acetylcholine was used as substrate throughout the present work. All cholinesterase measurements were made in the presence of 50 μM-ethopropazine, and with acetylcholine as substrate this concentration of ethopropazine inhibited 13·7% of the brain cholinesterase activity; the residual 86·3% was assumed to be the normal level of true cholinesterase in the brain and all values obtained after organophosphorus poisoning were expressed as a percentage of this.

Possibility of reversal of ethopropazine inhibition of pseudocholinesterase by oximes. It was not convenient to remove the oxime from reactivated

Table 1. *Inhibition of brain true cholinesterase and pseudocholinesterase by ethopropazine*

The cholinesterase (ChE) activity of 0·5 ml. of 'brain homogenate' was measured in the presence and absence of 50 μM-ethopropazine with 0·0138 M-acetylcholine (ACh), 0·03 M-acetyl-β-methylcholine (MCh) or 0·03 M-butyrylcholine (BuCh) as substrate. Temp. 37°; gas phase $N_2 + CO_2$ (95:5).

	ChE activity (μl. of CO_2/30 min./25 mg. of brain)		
Substrate ...	0·0138 M-ACh	0·03 M-MCh	0·03 M-BuCh
Control	121·0	66·5	18·0
+ ethopropazine	104·4	61·4	1·7
Inhibition (%)	13·7	7·7	91·5

Table 2. *Combined effect of oximes and ethopropazine on brain true cholinesterase (ChE) activity*

'Brain homogenate' (0·5 ml.) was kept at room temperature for 30 min. in the main compartment of a Warburg flask with: 50 μM-ethopropazine, mM-2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate (P2S) or 10 mM-monoisonitrosoacetone (MINA) and bicarbonate-saline buffer to 2·5 ml. The flasks were then equilibrated at 37° with $N_2 + CO_2$ (95:5) and the ChE activity was measured manometrically after adding 0·5 ml. of 0·0828 M-acetylcholine from the side arm. P2S, MINA and ethopropazine were omitted from the control.

Additions	ChE activity (μl. of CO_2/30 min.)	Inhibition (%)
Control	136·2	0
Ethopropazine	114·6	15·8
P2S	115·0	15·5
MINA	130·6	4·0
Ethopropazine + P2S	97·6	28·3
Ethopropazine + MINA	109·8	19·4

brain cholinesterase before measuring the enzyme activity, so that both oxime and ethopropazine were present during the activity determination. It was, therefore, important to be sure that pseudocholinesterase, inhibited by ethopropazine, would not be reactivated by the oxime. Table 2 shows that the inhibition of rat-brain cholinesterase by ethopropazine and 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate, or by ethopropazine and mono*iso*nitrosoacetone, was additive; inhibition by ethopropazine was therefore assumed not to be reversed by oximes.

Inhibition of cholinesterase by oximes and their possible interference in the activity tests. Since cholinesterase is inhibited by oximes (Hobbiger, 1956) it was necessary to ensure that the oxime, added to the homogenate for the reactivation, did not inhibit the cholinesterase in the subsequent activity test. The final concentrations of 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate and mono*iso*nitrosoacetone in the cholinesterase-activity determination were 0·167 and 1·67 mM respectively; reference to Fig. 1, in which percentage inhibition of true cholinesterase is plotted against the oxime concentration, shows that no correction was needed for these concentrations of 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate and mono*iso*nitrosoacetone.

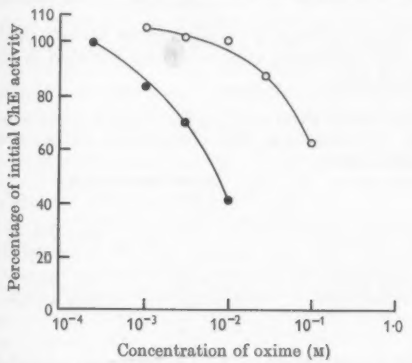

Fig. 1. Inhibition of brain true cholinesterase (ChE) activity by oximes. The reaction mixture contained: brain homogenate 0·5 ml., 0·028 M-$NaHCO_3$, 0·125 M-NaCl, 50 μM-ethopropazine, 0·0138 M-acetylcholine, and the stated concentrations of oxime, in a total volume of 3·0 ml.; pH 7·8, gas phase N_2+CO_2 (95:5). ○, Mono*iso*nitrosoacetone; ●, 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate.

Measurement of aging. As with erythrocyte cholinesterase (Blaber & Creasey, 1960) the percentage of aged cholinesterase was taken as the percentage of the initial activity which could not be recovered by oxime treatment; the ability to measure the proportion of aged brain cholinesterase, present at a particular instant, depends on the rate of the oxime-reactivation reaction being much greater than that of the aging process; with TEPP- and Sarin-inhibited cholinesterase this appeared to be so, as oxime treatment resulted in complete reactivation of the freshly inhibited enzyme within 1 hr. (Table 3). With DFP- and DMNP-inhibited brain cholinesterase, however, only partial reactivation of the freshly inhibited cholinesterase by oximes was observed, indicating that some aging had occurred either during, or before, reactivation by oximes. Thus although it was possible to say whether or not the aging of the DFP- or DMNP-inhibited brain cholinesterase was complete it was not possible to observe it as accurately as with TEPP- or Sarin-inhibited brain cholinesterase. Because 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate was the faster reactivator this oxime was used in all the routine measurements of aging.

Storage of brain preparations at 37°. It was impossible to store rat brain without some loss of activity. Homogenates showed a high initial loss of activity, approx. 11% in the first 24 hr., even in

Table 3. *Reactivation of inhibitied rat-brain cholinesterase by oximes*

A homogenate of rat brain inhibited *in vivo* with DMNP, TEPP, Sarin or DFP was allowed to stand at room temperature with mM-2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate (P2S) or 10 mM-mono*iso*nitrosoacetone (MINA). At suitable intervals the cholinesterase activity was determined and expressed as a percentage of the normal value.

Time of contact (hr.)	Percentage of normal cholinesterase activity							
	DMNP		TEPP		DFP		Sarin	
	P2S	MINA	P2S	MINA	P2S	MINA	P2S	MINA
0	17·2	17·2	24·9	12·4	31·6	31·6	31·8	31·8
0·5	87·0	36·6	98·5	93·5	65·5	56·4	97·3	96·1
1·0	88·0	42·6	100·0	98·4	77·6	68·0	100·0	100·0
2·0	88·1	63·3	100·0	100·0	80·9	77·2	100·0	100·0
3·5	88·0	70·6	—	100·0	89·0	86·5	—	—
4·5	93·7	80·4	—	—	—	—	—	—
5·0	—	—	—	—	85·4	85·6	—	—

Table 4. *Storage of half-brains*

Six freshly dissected normal rat brains were divided into *R* and *L* halves. The cholinesterase activity of the *L* halves was estimated immediately and the *R* halves stored at 37° in the presence of 8-hydroxyquinoline. The cholinesterase activity of the *R* halves was measured and expressed as a percentage of the corresponding *L* half at the intervals stated. Homogenate was stored at 37° in the presence of 8-hydroxyquinoline.

	Cholinesterase activity (μl. of CO_2/30 min./25 mg. of brain)				
	Intact half-brains			Homogenate	
Time (days)	*L*	*R*	$(R/L) \times 100$	(Units)	(% of initial activity)
1	103·8	105·7	100	108	100
2	97·7	97·3	100	96·5	89·4
3	105·8	102·0	96·5	—	—
4	108·4	100·8	93	79·4	73·5
7	109·4	56·3	51·5	—	—
8	97·5	82·6	84·8	—	—
13	—	—	—	63·9	59·2

the presence of preservatives such as 0·1% 8-hydroxyquinoline, 0·001% Merthiolate, or 0·08% Nipasept. However, when the half-brains were stored in tightly stoppered tubes in the presence of 0·1 ml. of 0·05% 8-hydroxyquinoline the cholinesterase activity remained unchanged for 48 hr.; during the following 6 days the activity gradually fell to approx. 85% of the initial value (Table 4). There was the occasional half-brain, e.g. Table 4, 7 days, which showed an abnormal loss of activity; in such cases the figures were rejected.

Interference from extracellular inhibitor. The assay procedures outlined so far were applied to homogenates prepared directly from the brains of poisoned animals. This practice has been criticized by Kewitz (1957) and by Kewitz & Nachmansohn (1957), who claimed that DFP and E600 could occur extracellularly in the brain, and that although they would not inhibit the cholinesterase *in vivo* they would do so *in vitro* as soon as the brains were homogenized in preparation for cholinesterase assay. Kewitz (1957) claimed that this interference could be prevented by vacuum-drying the brains and extracting with chloroform before homogenizing. Because of the doubt cast upon cholinesterase assays performed without Kewitz's extraction procedure it was necessary to find out whether the present cholinesterase assays would be invalidated in this way.

Some preliminary experiments showed that extraction of partially dried brains with chloroform resulted in complete loss of cholinesterase activity. In order to avoid this loss it was necessary to dry the brains for 3 days over P_2O_5 at 0·1 mm. Hg.

To investigate Kewitz's procedure further the behaviour of inhibited brain cholinesterase during the drying was studied in the following manner, where no chloroform extraction was performed. The brains from eight normal mice were divided into *R* and *L* halves; the *R* halves were pooled and weighed, and the mean cholinesterase activity (*a*) was estimated immediately. The *L* halves, after being weighed wet, were dried over P_2O_5 for 3 days at 0·1 mm. Hg and the mean cholinesterase activity after drying was expressed as a percentage of the initial activity of the *R* halves (*a*). Concurrently a similar experiment was performed on the brains from eight mice which had received an injection of 1 mg. of E 600/kg., the animals having been killed when signs of poisoning were well established. The mean cholinesterase activities of the pooled *R* half-brains before drying and of the pooled *L* half-brains after drying were again expressed as percentages of the initial cholinesterase activity (*a*) of the normal *R* half-brains.

Table 5. *Change in cholinesterase activity during the vacuum-drying of mouse brain inhibited* in vivo *with* E 600

The dose of E 600 was 1 mg./kg. body wt. intraperitoneally. All percentages refer to the initial activity of the control brain.

	Cholinesterase activity (μl. of CO_2/30 min./25 mg. wet brain)	
	Initial	After 3 days' drying
Control brain	112·5 (100%)	98 (87%)
Inhibited brain	45·9 (41%)	65·3 (58%)

The results (Table 5) show that during the drying the cholinesterase activity of the normal brains decreased to 87% of the control value whereas that of the poisoned brains increased by 17% of the control value, i.e. from 41 to 58%. This increase, obtained without chloroform extraction, indicates the spontaneous reactivation of 28·8% (i.e. $100 \times 17/59$) of the inhibited cholinesterase. The

increase in activity, observed by Kewitz & Nachmansohn (1957), after drying plus chloroform extraction, was equivalent to 32% of the inhibited enzyme. Thus spontaneous reactivation during the drying process could have accounted for the increase in cholinesterase activity which Kewitz & Nachmansohn attributed to the removal of extracellular inhibitor. Extracellular storage of inhibitor has, therefore, been assumed not to occur in the present experiments.

Recovery of brain cholinesterase activity in poisoned rats

In these experiments the rat-brain cholinesterase was inhibited *in vivo* by administering each inhibitor to a separate group of 72 animals. After inhibition the recovery *in vivo* and aging of the inhibited brain true cholinesterase was followed by performing cholinesterase assays before and after oxime treatment on a homogenate prepared from the pooled brains of batches of six rats taken at suitable intervals from each group. The cholinesterase activities were expressed as percentages of a normal value (*a*) previously determined in a group of 20 control rats. The percentage of aged enzyme was that percentage of the group normal (*a*) which could not be recovered by oxime treatment.

To follow the recovery *in vitro* of enzyme activity the brains of the first batch of six rats from each group were divided into R and L halves. The individual cholinesterase activities of the R halves, before and after oxime treatment, were measured immediately after removal from the animal. The L halves were stored at 37° in stoppered tubes with 8-hydroxyquinoline as preservative. For the first few days, whenever a batch of six rats was killed for brain cholinesterase measurements in the study *in vivo*, a stored L half-brain was also taken and its cholinesterase activity measured before and after oxime treatment. The activities so obtained were expressed as percentages of the group normal (*a*), and used to prepare the curve of recovery *in vitro*, a diagrammatic form of which is shown in Fig. 2.

Let the individual percentage cholinesterase activities of the R half-brains before oxime treatment $= R_1$ to R_6, and the mean of these activities $= R_m$; the individual percentage cholinesterase activities of the R half-brains after oxime treatment $= R_{0,1}$ to $R_{0,6}$, and the mean of these activities $= R_{0,m}$; the individual percentage cholinesterase activities of the stored L half-brains before oxime treatment $= L_1$ to L_6; the individual percentage cholinesterase activities of the stored L half-brains after oxime treatment $= L_{0,1}$ to $L_{0,6}$; corresponding R and L halves carry the same numerical subscript.

The mean values R_m and $R_{0,m}$ provided the first pair of points on both the *in vivo* and *in vitro* curves. Thus it was possible to follow the recoveries *in vivo* and *in vitro* from the same starting point and so achieve a fair comparison between the two. However, with Sarin, inhibition of the brain cholinesterase continued slightly after the first batch of rats was taken with the result that the curves of recovery *in vivo* and *in vitro* did not start from precisely the same cholinesterase level (Fig. 5).

The percentage of spontaneous reactivation which occurred during storage was given by the difference between the cholinesterase activities of the R and L halves of the same brain, i.e. $L_x - R_x$. The total cholinesterase activity after a particular period of storage, i.e. the uninhibited cholinesterase plus that produced by spontaneous reactivation, was given by $(L_x - R_x) + R_m$ and is the figure which is plotted on the spontaneous reactivation curve.

The percentage of non-aged cholinesterase present in the stored half-brains, i.e. the figure plotted on the aging curve, was given by the expression $(L_{0,x} - R_x) + R_m$, where $(L_{0,x} - R_x)$ is the difference between the cholinesterase activities of the oxime-treated stored L half-brain and the corresponding R half. $(L_{0,x} - R_x)$ includes the

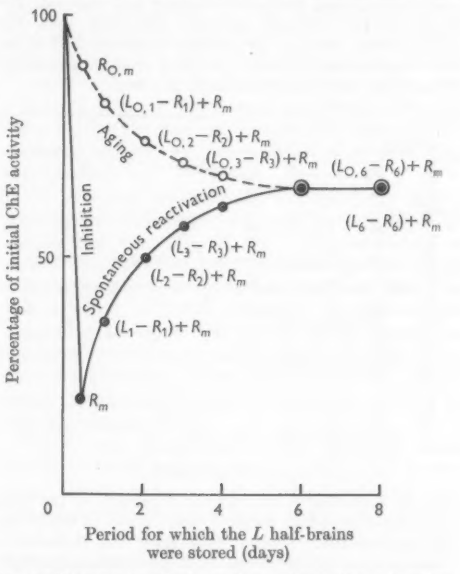

Fig. 2. Diagram to illustrate the method of constructing the curves showing the changes *in vitro* of rat-brain true cholinesterase (Che) activity after poisoning *in vivo* with organophosphorus cholinesterase inhibitors. For definition of letters see text.

increase in cholinesterase activity produced by spontaneous reactivation as well as that produced by oxime treatment.

The results of these experiments are shown in Figs. 3–6.

In vitro, significant spontaneous reactivation was observed only with the DMNP- and TEPP-inhibited cholinesterase. Aging, on the other hand, was observed with all the inhibitors used, as has been described with inhibited erythrocyte cholinesterase (Blaber & Creasey, 1960). When all the oxime-reactivatable cholinesterase had disappeared and only aged cholinesterase remained no further increase in DMNP-, DFP- or Sarin-inhibited cholinesterase activity was observed.

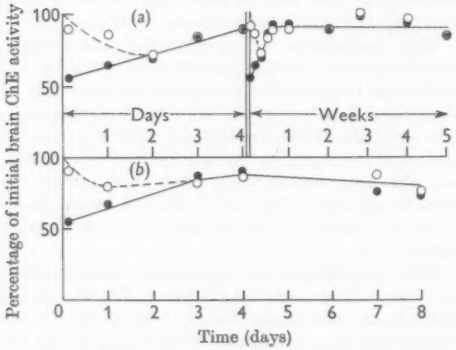

Fig. 3. Behaviour *in vivo* (*a*) and *in vitro* (*b*) of the brain cholinesterase of rats poisoned with 0·25 mg. of TEPP/kg. subcutaneously. Broken lines show the decrease in cholinesterase reactivatable by 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate and represent the aging of the inhibited enzyme. Full lines show the cholinesterase activity before oxime treatment and represent spontaneous recovery (*in vitro*) or spontaneous recovery plus new enzyme synthesis (*in vivo*).

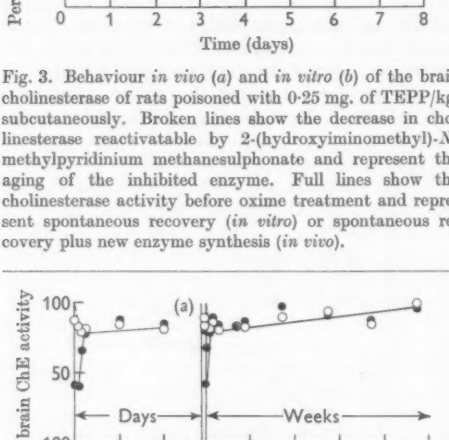

Fig. 4. Behaviour *in vivo* (*a*) and *in vitro* (*b*) of the brain cholinesterase of rats poisoned with 2·0 mg. of DMNP/kg. intraperitoneally. Broken lines show the decrease in cholinesterase reactivatable by 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate and represent the aging of the inhibited enzyme. Full lines show the cholinesterase activity before oxime treatment and represent spontaneous recovery (*in vitro*) or spontaneous recovery plus new enzyme synthesis (*in vivo*).

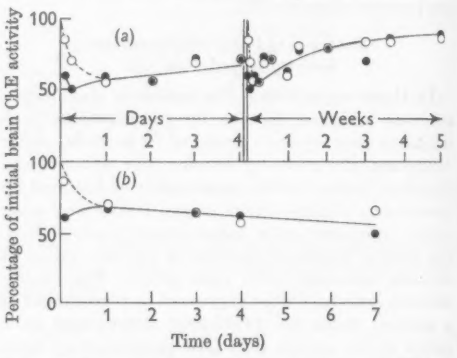

Fig. 5. Behaviour *in vivo* (*a*) and *in vitro* (*b*) of the brain cholinesterase of rats poisoned with 0·012 mg. of Sarin/kg. subcutaneously. Broken lines show the decrease in cholinesterase reactivatable by 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate and represent the aging of the inhibited enzyme. Full lines show the activity before oxime treatment and represent spontaneous recovery (*in vitro*) or spontaneous recovery plus new enzyme synthesis (*in vivo*).

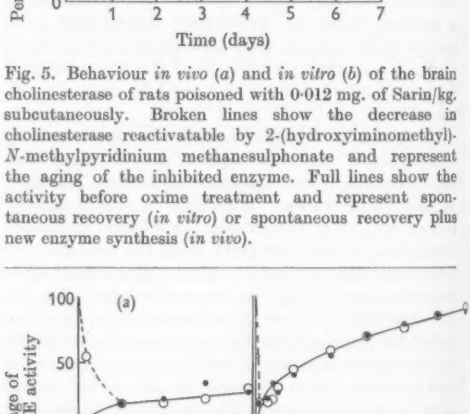

Fig. 6. Behaviour *in vivo* (*a*) and *in vitro* (*b*) of the brain cholinesterase of rats poisoned with 3·0 mg. of DFP/kg. subcutaneously. Broken lines show the decrease in cholinesterase reactivatable by 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate and represent the aging of the inhibited enzyme. Full lines show the cholinesterase activity before oxime treatment and represent spontaneous recovery (*in vitro*) or spontaneous recovery plus new enzyme synthesis (*in vivo*).

With TEPP-inhibited cholinesterase, however, a slight and unexplained increase in activity was observed after all oxime-reactivatable cholinesterase activity had disappeared (Fig. 3).

In vivo, spontaneous reactivation and aging of the inhibited cholinesterase occurred at rates similar to those observed *in vitro*. Spontaneous reactivation of the TEPP- and DMNP-inhibited cholinesterase occurred much faster than the simultaneous aging process; consequently very little aged enzyme was produced and it was impossible to measure the rate at which it was replaced. With Sarin and DFP, where spontaneous reactivation of the inhibited enzyme occurred to only a very slight extent, the recovery *in vivo* of inhibited brain cholinesterase activity proceeded at an exponential rate, and with DFP (Fig. 6) the half-life of the inhibited cholinesterase was approximately 14 days.

DISCUSSION

Kewitz & Nachmansohn (1957) suggested that the storage of injected E 600 at extracellular sites could seriously interfere with tissue cholinesterase estimations performed by orthodox methods. They based this suggestion on their observation that mouse brains or diaphragms poisoned *in vivo* by E 600 had a higher cholinesterase activity if they were dried *in vacuo* and extracted with chloroform before performing the cholinesterase assay than if this procedure were omitted. They considered that chloroform extraction removed extracellularly stored E 600 which would otherwise have caused further inhibition during homogenizing and so given a falsely low indication of the cholinesterase level present in the tissues of the intact animal. By using their chloroform-extraction technique Kewitz & Nachmansohn obtained an increase in cholinesterase activity over that of preparations not so treated equal to 32% of the inhibited cholinesterase activity.

The present work shows that mouse brains have to be dried *in vacuo* for 3 days before chloroform extraction can be used without risk of inactivating some of the cholinesterase. When only the drying procedure was applied to mouse brains poisoned *in vivo* with E 600 the inhibited cholinesterase underwent spontaneous reactivation to the extent of 28·8% of the inhibited enzyme activity. This increase in cholinesterase activity, almost equal in magnitude to that reported by Kewitz & Nachmansohn (1957), was obtained without chloroform extraction and was due solely to spontaneous reactivation of the inhibited enzyme during drying. It is possible therefore that this mechanism operated in Kewitz & Nachmansohn's experiments and could provide an alternative explanation of their results. This view is consistent with the fact that in the present experiments DFP-inhibited cholinesterase did not undergo spontaneous reactivation and this could easily explain Kewitz & Nachmansohn's failure to detect extracellular DFP.

Although the possibility that some inhibitor occurs at extracellular sites cannot definitely be excluded on the basis of these experiments, in view of the alternative explanation offered for Kewitz & Nachmansohn's results, this possibility would appear to be slight and in the present work it has been discounted.

As for inhibited erythrocyte cholinesterase (Blaber & Creasey, 1960) rat-brain cholinesterase inhibited with organophosphorus compounds recovered its activity *in vivo* in one or two stages depending on whether or not the inhibited enzyme underwent spontaneous reactivation. Immediately after its formation the inhibited enzyme began to age, i.e. it changed spontaneously into a form which could no longer be reactivated by oximes or undergo spontaneous reactivation. With TEPP- or DMNP-inhibited cholinesterase the aging was accompanied by spontaneous reactivation of the inhibited enzyme, the two reactions proceeding simultaneously and their relative rates determining the extent of the spontaneous reactivation. With DFP- or Sarin-inhibited cholinesterase spontaneous reactivation of the inhibited enzyme occurred to only a very slight extent. During this stage of its recovery the inhibited cholinesterase behaved the same *in vivo* as *in vitro*. When the inhibited enzyme was present entirely in the aged form no further recovery occurred *in vitro*, and *in vivo* the second and slower stage of the recovery was reached.

In vivo the recovery of the DFP- or Sarin-inhibited cholinesterase occurred throughout at an exponential rate (Fig. 6) which was very close to that calculated for the turnover rate of brain protein by Gaitonde & Richter (1956) and based on the turnover of [^{35}S]methionine. In view of the similarity between the rates of cholinesterase recovery and brain-protein turnover it seems likely that the recovery of the DFP- or Sarin-inhibited brain cholinesterase activity was due entirely to new enzyme synthesis.

When the cholinesterase was inhibited with TEPP or DMNP two stages in its recovery *in vivo* could clearly be seen. Most of the enzyme activity returned during the first stage so that only a small residue of irreversibly inhibited cholinesterase was left as a result of the aging process. It was not possible to measure the rate at which this small quantity of inhibited cholinesterase was replaced during the second stage but it is reasonable to assume that replacement was by new enzyme synthesis and that the rate was exponential.

This exponential rate of new enzyme formation is in marked, though not unexpected, contrast to the linear rate of the second stage in the recovery *in vivo* of erythrocyte cholinesterase.

The two forms of inhibited brain cholinesterase of different stabilities originally postulated by Davison (1955) are probably the freshly inhibited enzyme which can undergo spontaneous reactivation and the aged enzyme which cannot.

These results show that the mechanisms by which the activity of the brain cholinesterase is restored to normal after organophosphorus poisoning are precisely analogous to those which operate for inhibited erythrocyte cholinesterase, namely spontaneous reactivation and aging of the inhibited enzyme, and new enzyme synthesis.

SUMMARY

1. Recovery of tetraethyl pyrophosphate- or dimethyl *p*-nitrophenyl phosphate-inhibited rat-brain true cholinesterase *in vivo* occurred in two stages, a rapid partial recovery being followed by a slower, and probably exponential, return to normal activity.

2. With di*iso*propyl phosphorofluoridate- or *iso*propyl methylphosphonofluoridate-inhibited rat-brain true cholinesterase only one stage was observed in the recovery *in vivo* which occurred throughout at an exponential rate.

3. The first stage in the recovery is explained in terms of spontaneous reactivation and aging of the inhibited enzyme, and the second stage is correlated with new enzyme synthesis associated with the normal turnover of brain protein.

REFERENCES

Austin, L. & Berry, W. K. (1953). *Biochem. J.* **54**, 695.
Bayliss, B. J. & Todrick, A. (1956). *Biochem. J.* **62**, 62.
Blaber, L. C. & Creasey, N. H. (1960). *Biochem. J.* **77**, 591.
Davison, A. N. (1953). *Biochem. J.* **54**, 583.
Davison, A. N. (1955). *Biochem. J.* **60**, 339.
Frawley, J. P., Hagan, E. C. & Fitzhugh, O. G. (1952). *J. Pharmacol.* **105**, 156.
Gaitonde, M. K. & Richter, D. (1956). *Proc. Roy. Soc.* B, **145**, 83.
Hobbiger, F. (1956). *Brit. J. Pharmacol.* **11**, 295.
Hobbiger, F. (1957). *Brit. J. Pharmacol.* **12**, 438.
Kewitz, H. (1957). *Arch. Biochem. Biophys.* **66**, 263.
Kewitz, H. & Nachmansohn, D. (1957). *Arch. Biochem. Biophys.* **66**, 271.

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The Estimation of the Available Lysine in Animal-Protein Foods

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This paper describes the development of a procedure for the estimation in foods of the lysine units whose ϵ-amino groups will undergo the Sanger reaction with fluorodinitrobenzene. There is reason to think that those lysine units in the proteins of heat-processed foods whose ϵ-amino groups are bound to other groups, and so are unable to react, are likely also to be nutritionally 'unavailable' (Eldred & Rodney, 1946; Lea & Hannan, 1950; Henry & Kon, 1950). As a consequence of this, methods of analysis for lysine that begin with acid hydrolysis have been found to overvalue foods as sources of this amino acid.

Our experiments have given results suggesting that chemical estimations of 'fluorodinitrobenzene-available' lysine can give a more reliable indication of the value of animal-protein foods (Carpenter, Ellinger, Munro & Rolfe, 1957; Lea, Parr & Carpenter, 1960).

Lysine is often the factor limiting the protein quality of mixed diets for man or animals. Cereals, which are usually the staple energy source, also supply a large proportion of the protein and this cereal protein is seriously deficient in lysine. It is therefore of practical importance that the processing of high-protein foods should be controlled so that damage to the lysine is minimized. The procedure to be described has therefore been designed to be practicable for use in a routine analytical control laboratory.

Carpenter & Ellinger (1955*a*, *b*) used a simple procedure which proved useful for a range of animal materials, but it was affected significantly by interference from α-dinitrophenylarginine when applied to autolysed materials, and from dinitrophenol coming from breakdown of excess of dinitrofluorobenzene, if carbohydrate were present (Conkerton & Frampton, 1959). The interference was avoided by a modification (Bruno & Carpenter, 1957) in which methoxycarbonyl chloride was

used, but this treatment led to the unexpected development of a coloured histidine derivative (Carpenter, Jones & Mason, 1959).

The description of a procedure designed to overcome these difficulties, and the results of tests for interference, recovery and reproducibility with animal-protein foods, are given below. It seems from our own experiments and those of others (Baliga, Bayliss & Lyman, 1959; J. Mauron, personal communication) that the determination of chemically 'available lysine' in foods rich in carbohydrates and the nutritional significance of the figures obtained can involve some special difficulties.

EXPERIMENTAL

1-Fluoro-2:4-dinitrobenzene (FDNB) and methoxycarbonyl chloride were obtained from British Drug Houses Ltd., Poole, Dorset. The FDNB is used as a 2·5% (v/v) solution in ethanol, which is made up freshly for each determination because of the danger of its vesicant effects if spilt on the skin. It may be solid at room temperature but it can be measured out by holding the bottle in warm water and warming the pipette before use; accuracy is not essential as the reagent is used in excess.

The diethyl ether used was the 'laboratory-chemical' grade supplied by May and Baker Ltd., Dagenham, Essex, and was stored with metallic sodium before use. The pH 8·5 buffer was 8% (w/v) $NaHCO_3$–8% (w/v) Na_2CO_3 (19:1, v/v) with a final adjustment with NaOH or HCl as required.

The sample used as a reference standard of ε-dinitrophenyl (DNP)-lysine hydrochloride (1) was prepared by Dr M. McC. Barnes, Rowett Research Institute, Aberdeen, by the procedure of Porter & Sanger (1948); (Found: C, 38·7; H, 5·18; N, 15·3; Cl, 9·69. $C_{12}H_{16}O_6N_4,HCl,H_2O$ requires C, 39·3; H, 5·18; N, 15·3; Cl, 9·69%). The lysine unit in this molecule represents 39·9% of its weight. As a substandard in routine assays we used a larger sample of ε-DNP-lysine (2) (E. I. du Pont Co., Wilmington, Delaware, U.S.A.). By comparison of its extinction with that of the reference standard of the hydrochloride, it was estimated to contain the equivalent of 45·5% of lysine. Solutions of the two samples in N-HCl showed a constant extinction ratio at all wavelengths between 250 and 480 mμ, with the expected absorption maxima at 265 and 365 mμ (Sanger, 1949). For use as a working standard in the analytical procedure, 150–160 μM-solutions of standard 2 in N-HCl were stored in the dark.

α-DNP-arginine and α-benzoylhistidine methyl ester had been prepared by Dr K. Bailey and Dr F. Sanger respectively, both of the Department of Biochemistry, Cambridge.

Test materials. A sample of threefold re-crystallized bovine insulin (Boots Pure Drug Co. Ltd., Nottingham), as used earlier by Sanger (1945), was used as a reference material.

As an example of a partially hydrolysed protein that might give rise to special interfering products, 0·4 g. of zein (British Drug Houses Ltd.) was refluxed for 4 hr. with 6 ml. of 5N-HCl, then neutralized and the analytical procedure was started with the addition of 0·64 g. of $NaHCO_3$ instead of 8 ml. of $NaHCO_3$ solution.

Measurement of extinction. The extinction of the final solutions was measured in an SP. 1400 prism absorptiometer (Unicam Instruments Ltd., Cambridge) set at 435 mμ and with 1 cm. cells, with the instrument set to give zero extinction with water. Other workers have tested the procedure, with instruments with filters giving maximum transmission at approximately 435 mμ, with closely similar results.

Analytical procedure

The principle of the procedure is first to convert lysine residues, with reactive ε-NH_2 groups in the food proteins, into the yellow ε-DNP-lysine by treatment of the material with FDNB, followed by acid hydrolysis. Ether-soluble interfering compounds are removed by extraction and the extinction of the residual aqueous layer is measured. A blank value is obtained by treatment with methoxycarbonyl chloride and extraction of the ether-soluble lysine compound which results.

Stage 1. At least 50 g. of the material to be analysed was ground so that it passed a 1/50 in. sieve. Samples were taken for the determination of N in duplicate. Two portions, each containing an estimated 30–50 mg. of N, were then taken into round-bottomed flasks and to each was added 8 ml. of 8% (w/v) $NaHCO_3$. They were shaken gently to disperse the material and then left for 10 min. FDNB (0·3 ml.), previously dissolved in 12 ml. of ethanol, was added to each flask, which was then stoppered and shaken gently on a mechanical shaker for 2 hr. The stoppers were removed and the flasks stood in boiling water until there was no more effervescence, even on shaking. It may be checked that this point corresponds to a loss of weight of 10 g., i.e. the weight of ethanol added. 8·1N-HCl (24 ml.) was added immediately and the flasks were refluxed gently for 16 hr. with condensers adequate to prevent loss of HCl. The flasks were then disconnected after washing the condensers with water. (The condensers may still give a yellow colour on being placed in alkaline washing water, owing to the presence of dinitrophenol, a decomposition product of FDNB which is colourless in acid solution.) After the flasks had stood in ice–water for 1–2 hr. the contents were filtered through a paper such as Whatman no. 541 with water washings and the filtrate was made up to 200 ml. A portion of each filtrate was diluted again if necessary so that 2 ml. contained an estimated equivalent of 35–55 μg. of 'available lysine' from the original sample. (This usually involved a twofold to fivefold dilution.)

Stage 2. Portions (2 ml.) from each diluted filtrate were pipetted into each of two glass-stoppered tubes A and B, graduated at 10 ml., and a small conical flask C. The contents of the tubes were extracted twice with 5 ml. (approx.) portions of ether, the ether layers were discarded and the tubes were held in boiling water until effervescence from the residual ether ceased, and then they were cooled. Tube A was made up to 10 ml. with N-HCl and kept for the final readings.

Stage 3. The contents of flask C were titrated with 10% (w/v) NaOH, with phenolphthalein as indicator, and then discarded. The same volume of NaOH was then added to tube B, followed by 2 ml. of buffer solution, pH 8·5. Methoxycarbonyl chloride (0·045–0·055 ml.) was then added and the tube shaken vigorously to disperse and dissolve the compound. After 5–10 min. 0·75 ml. of conc. HCl was added, cautiously at first and with agitation to prevent the contents frothing over. The contents were

again extracted twice with 5 ml. of ether. (The ether washings were discarded in the standard procedure but they have been used for some of the studies reported below.) The residual ether in the aqueous layer was evaporated by standing the tube in boiling water, and the volume was made to 10 ml. with water.

Stage 4. The extinction coefficients of the contents of tubes A and B were measured in 1 cm. cells at 435 mμ (or with a filter having maximum transmission between 420 and 450 mμ if necessary). 'Reading A – reading B' was taken as the extinction due to ε-DNP-lysine, and was compared with the corresponding values obtained with 2 ml. of standard DNP-lysine solution passed through the procedure from stage 2 onwards, with omission only of the ether-washing in stage 2. The equivalent amount of lysine from the test food that has reacted with FDNB is calculated, with a suitable correction for losses due to hydrolysis where necessary (from the experimental results given below this appears to be multiplication by a factor of 1·09).

RESULTS

Possible interfering compounds. Starting from intact or hydrolysed proteins the only coloured, ether-insoluble DNP derivatives to be expected in the final solution at the end of stage 2 of the procedure are ε-DNP-lysine and α-DNP-arginine; the remaining coloured α-DNP-amino acids are ether-soluble and are removed in stage 2. The extinctions of these two compounds treated as in stage 2 (tube A) are shown in Table 1 (column A).

Treatment with methoxycarbonyl chloride, re-acidifying, extracting with ether and making each fraction to 10 ml. as in stage 3 gave the results shown in the column B. The third column X shows the extinctions obtained by evaporating the ether washings and redissolving the residues in 10 ml. of N-HCl with warming (see Bruno & Carpenter, 1957). As reported by Bailey (1957), the lysine colour is found in the ether-soluble fraction X, whereas the arginine colour remains in B, water-soluble, so that use of the reading A – B eliminates the arginine interference.

A sample of α-benzoylhistidine methyl ester was taken through the complete procedure and in addition the ether-soluble material after methoxycarbonyl chloride treatment was taken up in N-HCl (tube X). After hydrolysis, portions were taken to give final dilutions equivalent to 30 μM concentrations of the original material. It is seen from Table 1 that the histidine DNP derivative was colourless until coming into contact with methoxycarbonyl chloride. However, the colour was largely ether-soluble and A – B gave a value of only −0·005. This would correspond, for a protein containing 2% of histidine reacting in the same way, to an apparent 'lysine content' of −0·06%.

The colour of the histidine derivative was reduced by the final 7 min. warming period of the procedure used for tube X. More prolonged heating failed to destroy the colour completely.

Further tests with an intact zein preparation and a partially hydrolysed sample also gave apparent 'lysine' values of 0·02 and 0·10% respectively. (These compare with a range from 1·5 to 8·0% for the apparent available lysine contents of the food proteins assayed.) This suggests that interference from other amino acids, either free or combined, is small.

Recovery of added ε-dinitrophenyl-lysine. It is not, of course, possible to check recovery by adding pure lysine at the beginning of the procedure because this is converted with FDNB at an alkaline pH into the ether-soluble αε-di-DNP-lysine (Sanger, 1945). Recoveries have therefore been measured from additions of 20–30 mg. of ε-DNP-lysine (standard 2) after the addition of the FDNB in

Table 1. *Behaviour of zein and certain derivatives of lysine, arginine and histidine in the analytical procedure*

The estimated concentration of amino acids from zein after complete hydrolysis was based on its containing 8·8 moles of amino acid residues/kg. The X readings were obtained with the procedure described by Bruno & Carpenter (1957). With the histidine derivative the value in parentheses was obtained when the final heating period, introduced to ensure solution of the dried extract in N-HCl, was omitted.

		$E_{1\,cm}$ at 435 mμ			
			After methoxycarbonyl chloride treatment		
Point of entry into procedure	Material (with final amino acid concentration in tube A)	Final reading in N-HCl (A)	Ether-insoluble (B)	Ether-soluble (X)	Difference (A – B)
Stage 1	Blank run	0·002	0·003	0·007	−0·001
Stage 2	ε-DNP-lysine–HCl (1) (30 μM)	0·203	0·002	0·201	0·201
Stage 2	α-DNP-arginine (30 μM)	0·090	0·089	0·008	0·001
Stage 1	α-Benzoylhistidine methyl ester (30 μM)	0·002	0·007	0·103 (0·173)	−0·005
Stage 1	Zein (600 μM)	0·003	0·002	0·029	0·001
Stage 1	Partly hydrolysed zein (600 μM)	0·018	0·014	0·017	0·004

Table 2. *Recovery of ε-dinitrophenyl-lysine added during analysis of foods, immediately before the acid-hydrolysis stage*

The food (0·3 g.) was analysed for lysine, both before and after the addition of 20–30 mg. of ε-DNP-lysine.

Food	Recovery of added DNP-lysine (%)
Fish meal (FM14)	94
Meat meal (MM5)	94
Whale-meat meal (WM7)	92
Gelatin	92
Gelatin + 10% of ribose	92
None (DNP-lysine alone)	91

triplicate runs with the foods listed in Table 2. The same foods were assayed without the addition of the standard and recoveries were estimated by measurement of the extra lysine recovered. Runs were also made with no food present. There was good agreement between the replicates in each series. The mean recovery in all the experiments was 92%. If this is used as an estimate also of the loss of ε-DNP-lysine formed from test foods used in the procedure, values determined should be increased by a factor of 1·09 to correct for the loss. This has been done for all the values given below.

When the procedure was applied to insulin, with this correction the value obtained was 2·49 g. of lysine/16 g. of N, which is close to the theoretical value, based on the molecular structure (Sanger & Thompson, 1953) of 2·57 g./16 g. of N. The slightly low value is to be expected here because insulin is particularly rich (in contrast with the proteins of the common foods) in histidine. As explained above, this amino acid leaves a slight residual colour in tube B.

Reproducibility of results. A statistical examination has been made of the results obtained from application of the procedure to 11 different foods. Each food had been analysed on two or more occasions, and on each occasion the analysis had been run on replicate samples to give altogether 66 individual values. A preliminary study gave a pooled standard deviation for a single value within a run of 0·10 g./16 g. of N. However, the standard deviation tended to be larger where the mean values were also large and consequently a logarithmic transformation was used for the final analysis of variance. Variance between figures obtained on different occasions (based on 17 degrees of freedom) was significantly greater than that between values obtained on the same occasion (based on 32 degrees of freedom). It was concluded that mean values (each being, as usual, from two replicates) for two foods could be considered significantly different (at the 5% probability level) if one were at least 5% greater than the other, provided that the values were all obtained in the same run. Where the two means were obtained on different occasions the difference would have to be 8% to reach the same level of significance.

Typical values. The results obtained with 24 materials, including commercial products from slaughter-house animals, fish and whales, are set out in Table 3. The values show a wide range, from 8·6 g./16 g. of N for a sample of spray-dried blood meal down to 1·5 g./16 g. of N for a sample of feather meal. There was also a wide range in some cases between individual samples sold under the same name; for example, the two whale-meat meals gave values of 3·2 and 7·0 g./16 g. of N.

Effect of modifications of method. Table 4 sets out a comparison between the results obtained by the new procedure for ten of the same materials and the corresponding results obtained by variations of the procedure. Variation I involves following the same procedure but omitting the blank (tube B) for both unknown and standard. For eight of the ten samples the modification had the effect of increasing the values by 8–14%; for whale-meat meal, the increase was only 4% (a value checked on three occasions). For the last sample (feather meal) it was 17%, but here the absolute value was low. The ranking of the materials in order of lysine content was not altered significantly by changing to variation I.

Variation II followed the procedure (Bruno & Carpenter, 1957) for which results with pure compounds are already given under column X in Table 1, except that the final extinctions were read at 440 instead of 435 mμ. The results are also increased by the same factor (1·09) in all three variations, as an allowance for losses in the acid-hydrolysis stage. The results are again higher than with the new procedure by an average of 13%. This was to be expected because of the interference of histidine in this determination. However, there is only one change from the original ranking.

For variation III, the extracts for variation II were read at both 400 and 440 mμ, and an adjustment was made on the basis that the histidine derivative gives a ratio of extinction at these two wavelengths of 2·85:1 and the lysine derivative of 1·57:1 (Carpenter *et al.* 1959). The formula used was:

$$\text{Percentage of 440 m}\mu\text{ colour due to lysine} = \frac{2{\cdot}85\,E_{440} - E_{400}}{(2{\cdot}85 - 1{\cdot}57)\,E_{440}} \times 100.$$

The results here were, on the average, 3% lower than those found by the new procedure but the ranking of the ten samples by this variation was unchanged.

Table 3. *Values of available lysine in animal products determined by the new procedure*

Samples coded FM, MM or WM with a number following had been distributed as part of a collaborative investigation of laboratory methods of protein-quality evaluation under the auspices of the Agricultural Research Council (Zuckerman, 1959). Samples 2 and 3 of dried pork were received from Mr D. S. Miller (Human Nutrition Research Unit, Mill Hill, London, N.W. 7), sample 5 of ossein from Dr J. E. Eastoe (British Gelatin and Glue Research Association, Holloway, London, N. 7) and sample 18 of freeze-dried cod fillets from Mr E. J. Rolfe (Ministry of Agriculture, Fisheries and Food Experimental Factory, Aberdeen). The remaining materials were commercial ones for which the origins were unknown.

Sample no.	Material analysed	Available lysine (g./16 g. of N)
1	Blood meal, spray-dried	8·6
2	Pork meat, freeze-dried	7·4
3	Pork meat, after 24 hr. at 105°	5·3
4	Gelatin	4·5
5	Ossein	3·4
6–7	Meat meals with > 13% of N (MM3; MM5)	4·1; 4·1
8–10	Meat meals with 9–10% of N (MM16; MM18; A)	2·7; 5·3; 5·2
11–12	Meat meals with < 8% of N (MM10; B)	3·9; 4·1
13	Bovine insulin	2·5
14	Hoof-and-horn meal	1·9
15	Feather meal (MM20)	1·5
16–17	Whale-meat meals (WM7; WM9)	3·2; 7·0
18	Cod fillets, freeze-dried	8·4
19	Herring meal, stored under controlled conditions	7·2
20	Herring meal, from a bulk store in which there had been spontaneous heating	5·2
21–23	Fish meals, mixed species (FM14; FM15; E)	6·6; 6·4; 6·9
24	Condensed fish solubles	4·0

Table 4. *Comparison of values obtained by the new procedure and by three variations*

Variation I consists of the standard procedure with the omission of a blank correction (tube B); II is based on the colour (read at 440 mμ) of the material extracted from the blank (Tube B) in the standard procedure; III is based on II with a correction for the interference of a histidine derivative, based on the relative extinctions of both the histidine and lysine derivatives at 400 and 440 mμ. The samples are given the numbers used in Table 3, where their descriptions can be seen.

Sample no.	Values obtained for available lysine (g./16 g. of N)			
	New procedure	I	II	III
2	7·4	8·0	8·7	7·5
19	7·2	8·0	7·8	7·3
17	7·0	7·3	8·1	6·9
21	6·6	7·5	7·3	6·5
22	6·4	7·3	6·8	6·1
3	5·3	5·7	6·3	5·2
10	5·2	5·9	5·7	5·0
12	4·1	4·7	4·4	3·9
16	3·2	3·6	3·9	3·1
15	1·5	1·7	1·6	1·4

The development of a coloured histidine derivative from the application of variation II to α-benzoylhistidine methyl ester was almost entirely prevented by treatment of the hydrolysate with sodium sulphide before the addition of methoxycarbonyl chloride (Carpenter *et al.* 1959). It has not, however, proved possible to obtain consistent results when this pretreatment has been applied to analyses of food materials.

DISCUSSION

The usual application of the Sanger (1945) reaction of FDNB with free amino groups in proteins has been to study the molecular structure of purified materials. This is an attempt to adapt the usual procedure so that it could be applied even to crude foodstuffs and yield results indicating one aspect of their nutritive value, with only the skill and facilities to be found in a routine analytical laboratory.

Even the simple colorimetric measure of the ether-washed acid-hydrolysates of a range of animal-protein foods treated with FDNB gave results correlating well with the value of these foods as supplements to cereals in assays with chicks (Carpenter & Ellinger, 1955*a*, *b*). These results, and also similar results obtained later, have been encouraging for the possibilities of an analytical approach of this type.

The conditions of Sanger (1945) for the reaction with FDNB have been adopted. However, for simplicity and because of the possible presence of small peptides giving soluble DNP-peptides, the DNP-protein was not separated before the acid-hydrolysis stage. Ethanol was removed by evaporation and then the whole digest was acidified and

refluxed. Surprisingly, the presence of excess of FDNB and the non-protein components of the food does not seem to result in additional losses. Of ε-DNP-lysine added at the beginning of the hydrolysis stage, approximately 92% has been recovered in runs with a range of foods. This is in agreement with the findings of Sanger (1945) and Porter & Sanger (1948), who obtained recoveries of 90–95% under rather similar conditions with purified DNP-proteins freed from excess of FDNB, and concluded that ε-DNP-lysine was one of the most stable of the DNP-amino acids. Solomons & Irving (1958) found similar recoveries in their work with collagen, but, in contrast, Partridge & Davies (1955) have concluded that with hydrolysis of DNP-elastin there are large losses of ε-DNP-lysine, and that even with specially modified conditions only 65% of the added compound is recovered. Certainly there has been no such result in our own series of tests, but the results with elastin do suggest that further recovery trials would be advisable whenever a new type of foodstuff is being investigated.

Most of the coloured DNP-amino acids that might be present in our hydrolysates are removed by ether-extraction, but, in addition to ε-DNP-lysine, the aqueous residue may contain α-DNP-arginine, δ-DNP-ornithine, ε-DNP-hydroxylysine and water-soluble breakdown products from other DNP-amino acids and interaction products from the excess of FDNB and the non-protein components of the foods being tested (cf. review by Levy, 1955).

Use of the Bailey (1957) reaction with methoxycarbonyl chloride decreases interference in the standard procedure to those molecules that change (like ε-DNP-lysine) to being ether-soluble with this treatment, or which remain water-soluble and change their extinction. α-DNP-arginine is unchanged. The colourless DNP-histidine derivative becomes coloured after treatment with methoxycarbonyl chloride and a small proportion remains water-soluble, but for ordinary foods this should not decrease the true lysine value by more than 0·05 g./16 g. of nitrogen. Any reactive ornithine is measured in full, but it has not been considered a likely component of the foods considered here. Hydroxylysine would also be measured, although it cannot replace lysine as a dietary essential amino acid. In bone, tendon and skin collagens, hydroxylysine constitutes 12–21% of the total 'lysine plus hydroxylysine' present (Eastoe, 1955), and the two components react equally with FDNB through their ε-amino groups (Solomons & Irving, 1958). Despite this source of interference, the presence of collagen in a food will not give a misleading impression that it is of high quality as a source of lysine, because even the level of 'lysine plus hydroxylysine' is considerably lower than that of the lysine in undiluted and undamaged-muscle protein.

The procedure does not measure lysine units with both their α- and ε-amino groups free, although these units are nutritionally active. With FDNB they yield di-DNP-lysine, which is ether-soluble. Where the procedure is used as a form of quality control this can actually be an advantage since most animal-protein foods that have been carefully prepared contain only very low amounts of free amino acids; the use of stale raw material in which there had been appreciable autolysis would result in lower values' being recorded. Where it is required to include free lysine in the measurement, the preliminary addition of copper carbonate to block the free α-amino groups (Porter & Sanger, 1948; Solomons & Irving, 1958) may be a suitable modification, but we have not studied the effect of this step on recoveries and interference at later stages.

The application of the procedure to a range of foods has given consistently reasonable results. No figure higher than the estimated total lysine content of each food has been obtained. Low values have been associated with heat-damage. There is no evidence of proteins' remaining undenatured, and ε-amino groups' being unreactive for that reason, in the carefully dried products.

The two whale-meat meals listed in Table 3, WM7 and WM9, gave very different values (3·2 and 7·0 g./16 g. of nitrogen respectively). Bunyan & Price (1960) have found 8·1 g. of total lysine/16 g. of nitrogen in WM7, but the inferior quality of this sample is indicated by their further finding that its protein had a biological value for rats of only 32, as compared with a value of 69 obtained for WM9. Herring meals, from stores in which there had been heating as a result of the auto-oxidation of their unsaturated fat, also gave low values, as illustrated by sample 20 in Table 3. This has again been paralleled by a fall in nutritive value (Lea *et al.* 1960).

A comparison of results obtained by the new procedure with those obtained by three slightly different procedures (described here as variations I, II and III) that had been used earlier suggested that although they contained known sources of possible error the ranking of samples of the types tested would be little affected by the use of one procedure rather than another. Variation I has given values from 8 to 14% higher than with the new procedure for fish and meat meals in the present series. The results reported by Lea *et al.* (1960) were obtained by this procedure but had not been multiplied by 1·09 to correct for hydrolysis losses, so that we would expect them to range from 99 to 105% of the values that would be obtained by the new procedure with the correction factor applied.

Variation II, based on the previously ether-insoluble colour that becomes ether-soluble with methoxycarbonyl chloride, also gives high results. Bunyan & Price (1960) used this in its original form with no correction for destruction during hydrolysis (Bruno & Carpenter, 1957), and report values for six samples coded WM and FM that also appear in Table 3; their results are similar to ours and the samples have the same ranking in each case.

In view of the wide differences between the mean values obtained for different samples, the reproducibility of the procedure, as calculated from a statistical analysis of a large number of results, seems adequate.

SUMMARY

1. The Sanger reaction with 1-fluoro-2:4-dinitrobenzene for the determination of the free ϵ-amino groups of lysine units in purified proteins has been applied to animal-protein foods in an attempt to measure nutritional damage which may occur through the formation of enzyme-resistant linkages with ϵ-amino groups during processing.

2. The dinitrophenyl-proteins were hydrolysed with acid without separation from the digest, and the hydrolysates were ether-extracted. The measure of ϵ-dinitrophenyl-lysine was based on the further decrease in colour in the aqueous layer after digestion with methoxycarbonyl chloride and re-extraction with ether. This procedure gave a nil value for zein and the expected value for insulin.

3. Twenty-four materials, prepared in most cases as human or animal foods, gave a wide range of values from 1·5 g./16 g. of nitrogen for feather meal to 8·4 and 8·6 g./16 g. of nitrogen for freeze-dried cod fillets and spray-dried blood meal respectively. Recovery of added ϵ-dinitrophenyl-lysine was 92%.

4. Samples that had been heated and had shown decreased nutritional value in feeding tests also showed lower 'available lysine' values in the chemical procedure.

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REFERENCES

Bailey, K. (1957). *Biochem. biophys. Acta*, **24**, 162.
Baliga, B. P., Bayliss, M. E. & Lyman, C. M. (1959). *Arch. Biochem. Biophys.* **84**, 1.
Bruno, D. & Carpenter, K. J. (1957). *Biochem. J.* **67**, 13P.
Bunyan, J. & Price, S. A. (1960). *J. Sci. Fd Agric.* **11**, 25.
Carpenter, K. J. & Ellinger, G. M. (1955*a*). *Biochem. J.* **61**, xi.
Carpenter, K. J. & Ellinger, G. M. (1955*b*). *Poult. Sci.* **34**, 1451.
Carpenter, K. J., Ellinger, G. M., Munro, M. I. & Rolfe, E. J. (1957). *Brit. J. Nutr.* **11**, 162.
Carpenter, K. J., Jones, W. L. & Mason, E. L. (1959). *Biochem. J.* **73**, 11P.
Conkerton, E. J. & Frampton, V. L. (1959). *Arch. Biochem. Biophys.* **81**, 130.
Eastoe, J. E. (1955). *Biochem. J.* **61**, 589.
Eldred, N. R. & Rodney, G. (1946). *J. biol. Chem.* **162**, 261.
Henry, K. M. & Kon, S. K. (1950). *Biochem. biophys. Acta*, **5**, 455.
Lea, C. H. & Hannan, R. S. (1950). *Biochem. biophys. Acta*, **4**, 518.
Lea, C. H., Parr, L. J. & Carpenter, K. J. (1960). *Brit. J. Nutr.* **14**, 91.
Levy, A. L. (1955). *Meth. biochem. Anal.* **2**, 359.
Partridge, S. M. & Davies, H. F. (1955). *Biochem. J.* **61**, 21.
Porter, R. R. & Sanger, F. (1948). *Biochem. J.* **42**, 287.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Sanger, F. (1949). *Biochem. J.* **45**, 563.
Sanger, F. & Thompson, E. O. P. (1953). *Biochem. J.* **53**, 366.
Solomons, C. C. & Irving, J. T. (1958). *Biochem. J.* **68**, 499.
Zuckerman, S. (1959). *Nature, Lond.*, **183**, 1303.

Biochem. J. (1960) **77**, 610

The Intracellular Distribution of Glycolytic and other Enzymes in Rat-Brain Homogenates and Mitochondrial Preparations

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It has been shown (Hers, Berthet, Berthet & de Duve, 1951) that, although most of the liver hexokinase is soluble, a proportion of the hexokinase of various mammalian tissues is associated with subcellular particles (Long, 1952; Crane & Sols, 1953), and the latter workers have shown that with brain almost all of the enzyme is particulate. Glycolytic activity has been reported for various brain mitochondrial preparations (Du Buy & Hesselbach, 1956; Gallagher, Judah & Rees, 1956; Balázs & Richter, 1958; Abood, Brunngraber & Taylor, 1959). It seemed of interest therefore to determine the

distribution of each enzyme of the glycolytic chain in brain fractions and to examine the homogeneity of brain mitochondrial preparations.

METHODS

Special chemicals and reagents. The following chemicals were obtained from the sources indicated: glucose, glucose 6-phosphate (barium salt), phenyl butyrate, collidine and triethanolamine (British Drug Houses Ltd.) (the collidine and triethanolamine were both fractionally distilled before being converted into hydrochlorides for use as buffers); sodium pyruvate and acetylcholine chloride (Roche Products Ltd.); 2-deoxyglucose (L. Light and Co. Ltd.); ethylenediaminetetra-acetic acid (EDTA; Hopkin and Williams Ltd.; the potassium salt was used at all times); bovine-serum albumin (fraction V) (The Armour Laboratories); 2:3-diphosphoglyceric acid (barium salt; Schwarz Laboratories Inc., U.S.A.); DL-glyceraldehyde 1-bromide 3-phosphate (dioxan addition compound); and 'cyclohexylammonium dihydroxyacetone phosphate dimethyl ketal' (California Corporation for Biochemical Research, U.S.A.) (free dihydroxyacetone phosphate was prepared from its complex by conversion into free acid on a deionizing column followed by hydrolysis of the ketal at 40° for 4 hr.); heavy water (D_2O) (Imperial Chemical Industries Ltd.); Ficoll (Atkieselsekabt Pharmacia, Copenhagen; the compound is a high-molecular-weight carbohydrate of unspecified composition; a 20% soln. was dialysed against cold distilled water for 20 hr. before use); adenosine triphosphate (ATP), disodium salt (Sigma Chemical Co., U.S.A.); reduced diphosphopyridine nucleotide (DPNH), disodium salt; adenosine diphosphate (ADP), trisodium salt; barium salts of fructose 6-phosphate, phosphopyruvic acid, DL-2-phosphoglyceric acid and DL-3-phosphoglyceric acid; and an equimolar solution (0·1 M) of sodium triose phosphate esters (C. F. Boehringer und Söhne, Mannheim, Germany). Barium salts were converted into free acids by using Amberlite IR-120 resin, and the acids were neutralized with KOH: an arbitrary allowance of 30% was made for loss by adsorption on the resin. Purified enzymes were obtained as follows: glucose 6-phosphate dehydrogenase (Sigma Chemical Co., U.S.A.) (anhydrous type III was substantially free of hexokinase but contained some phosphoglucose isomerase); all other purified enzymes were supplied as suspensions in ammonium phosphate solution (C. F. Boehringer und Söhne, Mannheim, Germany).

Butyrylcholine perchlorate was prepared according to Aldridge (1953). Triphosphopyridine nucleotide (TPN) was supplied by Mr C. J. Threlfall and was about 80% pure.

Fractionation procedure. Brains from two decapitated rats (mature females of the Porton strain) were homogenized in 0·3 M-sucrose with a smooth glass tube and a Perspex pestle with all-round clearance of 0·005 in. (Aldridge, 1957). The method of preparing washed fractions by centrifuging, and the nomenclature applied to them, have been defined by Aldridge & Johnson (1959). For most experiments, the homogenate was divided by centrifuging at 20 000 *g* for 30 min. into one washed fraction (20.particles.30), containing cell debris, nuclei, mitochondria and some microsomes, and a supernatant (S) containing soluble proteins and some microsomes. In a few cases a more extensive fractionation was carried out giving cell debris plus nuclei (N), mitochondrial (M_1 and M_2), microsomal (P) and supernatant (S) fractions. The prefixed numbers are $10^{-3} \times$ the sedimentation force (*g*) and the suffix is the sedimentation time in minutes. Each particulate fraction was washed once by resuspending in 0·3 M-sucrose and sedimenting under the same conditions as those used for preparing the fraction: the washings were added to the supernatant before separation of the next fraction.

Fractionation in dense media. A homogenate was prepared as above. An unwashed nuclear fraction (1.N.10) was discarded, and an unwashed mitochondrial pellet (13.M.15), was obtained from the remainder; the fraction was subfractionated on a density gradient by one of two methods with the Spinco model L centrifuge.

In procedure A, which was used for preliminary studies, 4·0 ml. portions of the material suspended in 0·3 M-sucrose were layered over 1·4 ml. portions of a dense medium in each of three centrifuge tubes fitting the swing-out head (SW 39) of the Spinco. The tubes were centrifuged at maximum speed (40 000 rev./min.) for 1 hr. (145 000 *g* at the bottom of the tubes). Some material was sedimented into a pellet at the bottom of the tube, and some was retained at the interface. The fractions were carefully separated with a Pasteur pipette and resuspended in EDTA solution (potassium salt, 2 mM, pH 7·0); the clear liquid above each layer of particles was removed separately and retained: it was shown that the clear liquids contained very little of the enzymes being studied.

Procedure B was devised to enable larger quantities to be handled in one tube, and to avoid the difficulties associated with an interface. The crude mitochondrial pellet was suspended evenly in about 20 ml. of a suitable dense medium and placed in a 30 ml. centrifuge tube to fit the no. 30 angle head of the Spinco; the remaining tube-space was filled with 0·3 M-sucrose to prevent collapse of the walls. The tube was then centrifuged at maximum speed (30 000 rev./min. 105 000 *g* at the bottom of the tube) for 1 hr. The particles separated into a packed pellet at the bottom and a particle layer at the top of the dense solution, while a small proportion, having the same density as the solution, remained suspended; no soluble or particulate enzymes migrated into the 0·3 M-sucrose. Since the centrifugal field was not directly down the axis of the tube, a small amount of the dense particles which should have been sedimented to the bottom, adhered to the side wall of the tube: as the subfractionation was a quite arbitrary process, it was found convenient to combine all the unsedimented material into one subfraction, and the sedimented particles including those adhering to the wall into the other subfraction. Although having certain advantages, this method obviously cannot be adapted to the separation of more than two subfractions, whereas, if sufficiently delicate sampling devices and assay procedures are available, procedure A can be used to separate multiple subfractions.

Most studies with density gradients have utilized high concentrations of sucrose (Kuff & Schneider, 1954), but colloidal silica (Mateyko & Kopac, 1959), high-molecular-weight polymers (Thomson & Klipfel, 1958; Holter & Moller, 1958) and heavy water (Beaufay, Bendall, Baudhin, Wattiaux & de Duve, 1959) have also been used. The aim of the later procedures has been to decrease the possible damaging effects of high concentrations of sucrose. It is almost impossible to obtain full activity of succinic dehydrogenase in brain mitochondria, which have been sedimented

through 1·0 M-sucrose, even if the particles are subsequently suspended in water for 2 hr.: the latter, or similar treatment to cause damage to the mitochondrial membrane, was necessary and effective for the assay of succinic dehydrogenase in mitochondria (Aldridge & Johnson, 1959). The use of heavy water to increase the density of the medium is based on the presumption that, under the working conditions, exchange of the water within the particles for heavy water is not rapid. Beaufay *et al.* (1959) have shown that this is probably so for liver-mitochondria preparations, but is less true for lysosomes. Of the available high-molecular-weight polymers, Ficoll, used by Holter & Moller (1958), seems to have the required negligible osmotic pressure in solution combined with stability and moderate viscosity; this material, dissolved in 0·3 M-sucrose, was chosen for most experiments with procedure B. Sucrose solutions were usually used in procedure A where the stickiness of Ficoll solutions caused particles retained at the interface to cohere, forming a mass difficult to disperse.

Spectrophotometric assay methods

Enzyme activity was determined by the rate of change of concentration of reduced pyridine nucleotide, as shown by extinction measurements at 340 mμ. Where it was necessary to couple the reaction being assayed to a reaction involving pyridine nucleotide, purified enzymes were added in excess. Tissue suspensions were stored at 0°, and diluted to a suitable volume just before assay. Tubes containing all the assay-medium components except coupling enzymes and tissue were prepared: 0·05 ml. of coupling enzyme was added where necessary, followed immediately by 0·1 ml. of the dilute tissue suspension, making a final volume of 3·0 ml. The mixture was tipped into a 1 cm. quartz cell, and optical readings were taken against a blank at 20–30 sec. intervals over a period of about 8 min. with a Unicam SP.700 spectrophotometer. The blank contained the basic medium plus tissue but omitted substrate, DPNH and coupling enzymes. Preliminary control experiments were performed with each assay to show that the coupling enzyme was not contaminated with the enzyme being assayed, and that the substrate was not reacting with DPNH by any route other than that being studied. A suitable control was then run in parallel with each determination and consisted of the basic medium plus DPNH and tissue suspension; substrate and coupling enzymes were omitted. The control recorded oxidation of DPNH by endogenous tissue enzymes and substrates, and by air. Direct reading of the sample against control was inconvenient. In all except the α-glycerophosphate dehydrogenase and phosphofructokinase assays, tissue suspension equivalent to less than 1 mg. of original brain was needed, and the control rate was less than 10% of the sample rate. After sample readings had been corrected for control changes, the extinctions were plotted against time and the rate was measured from the best straight line. For some, the rate was irregular during the first 100–150 sec., and then became linear until most of the pyridine nucleotide had been used. Rates of change of extinction of 0·05/100 sec. over the range 0·6–0·2 were convenient in most cases, and were linear with respect to concentration of enzyme. Temperature was uniform during each experiment and varied during the series over the range 19–23°. Specific activities of whole homogenate are reported ±deviation for two determinations, and ±standard deviation for more than two. One extinction unit is an extinction of 1·00 under the described conditions.

The enzymes of the glycolytic chain are grouped below according to the final reaction to which they have been coupled.

Assay coupled to glucose 6-phosphate dehydrogenase. Phosphoglucose isomerase was assayed by conversion of the glucose 6-phosphate produced into 6-phosphogluconic acid with concomitant reduction of TPN. The method is essentially that of Slein (1955). The 3 ml. reaction medium contained (in final concentration and at pH 7·4) triethanolamine buffer (50 mM), KCl (10 mM), $MgCl_2$ (10 mM), EDTA (2 mM), potassium fructose 6-phosphate (3 mM), TPN (0·2 mM), glucose 6-phosphate dehydrogenase coupling enzyme and tissue. Slow rates were measured in order that the minimum amount of coupling enzyme should be used; the control rates due to phosphoglucose-isomerase contamination of the coupling enzyme were then 40% of the total rates measured, making the method of only moderate accuracy. The specific activity of whole homogenate was 5 extinction units/100 sec./g. of brain (one experiment).

Assays coupled to α-glycerophosphate dehydrogenase. Phosphofructokinase, aldolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase were assayed by oxidation of DPNH accompanying the conversion of dihydroxyacetone phosphate into α-glycerophosphate.

(*i*) Assay of phosphofructokinase. The method is based on that of Racker (1947), but with increased ATP concentration and addition of cysteine to stabilize the enzyme.

The 3 ml. reaction medium contained (in final concentration and at pH 7·6) ammonium phosphate (3 mM), $MgCl_2$ (2 mM), cysteine hydrochloride (7 mM), potassium fructose 6-phosphate (7 mM), ATP (0·44 mM), DPNH (80 μM), aldolase and a mixture of α-glycerophosphate dehydrogenase and triose phosphate isomerase coupling enzymes, and tissue. The higher ATP concentration enabled maintenance of a steady rate for a longer period, and also discouraged any tendency for contaminating glyceraldehyde phosphate dehydrogenase to divert the coupling reaction with reduction of DPN. The control rate was high (25% of the total), probably owing to the comparatively large amounts of tissue needed: addition of 3 mM-KCN did not decrease the control rate. The enzyme is unstable but was preserved for 1–2 hr. in sucrose solution at 0° in the presence of 0·5% of albumin buffered with 0·05 M-glycylglycine at pH 7·0. The rate plots were erratic and not reliable to more than 10%; a rate of change of extinction of not more than 0·04/100 sec. gave the best results. The specific activity of whole homogenate was 15±1 extinction units/100 sec./g. of brain (two experiments).

(*ii*) Assay of aldolase. The method is essentially that of Baranowski & Niederland (1949), but the medium chosen was identical with that used in the assay of phosphofructokinase, except that the substrate was sodium hexose diphosphate, and α-glycerophosphate dehydrogenase and triose phosphate isomerase were used as coupling enzymes. Rates of change of extinction up to 0·10/100 sec. were linear with respect to tissue concentration, and the control rate was less than 10% of the sample. The crude enzyme was stable in sucrose homogenates for at least 24 hr. at 0°. The specific activity of whole homogenate was 45±1 extinction units/100 sec./g. of brain (two experiments).

(*iii*) Assay of triose phosphate isomerase. The method is essentially that reported by Beisenherz (1955), but with increased reactant concentrations to allow a longer duration

of steady reaction. The 3 ml. reaction medium contained (in final concentration and at pH 7·4) triethanolamine buffer (50 mM), DL-glyceraldehyde 1-bromide 3-phosphate (0·6 mM, D-form, dioxan complex), DPNH (80 μM), α-glycerophosphate dehydrogenase coupling enzyme, and tissue. (The bromide is rapidly hydrolysed in solution but neither the bromide ion nor the dioxan interfered with the assay.) Rates of change of extinction up to 0·10/100 sec. were linear with respect to tissue concentration, and control rates were negligible. Rates were unaltered in the presence of potassium iodoacetate (3 mM), showing that there was no interference from glyceraldehyde phosphate dehydrogenase. Such high rates of reaction were obtained that tissue dilutions were performed with 0·3 M-sucrose containing 0·5% of albumin to hinder denaturation of the enzyme at the great dilutions necessary. The specific activity of whole homogenate was 2320 ± 120 extinction units/100 sec./g. of brain (two experiments).

(*iv*) Assay of diphosphopyridine nucleotide-dependent α-glycerophosphate dehydrogenase. The method is that described by Beisenherz, Bücher & Garbade (1955), and the medium is that used in the triose phosphate isomerase assay, except that no coupling enzyme is necessary. The substrate, dihydroxyacetone phosphate (0·6 mM), is accompanied by either an equimolar amount of glyceraldehyde 3-phosphate or by methanol according to the source of supply (see Special chemicals and reagents): these additions cause no interference, for the control rate was zero both in the presence and absence of iodoacetate (3 mM), and each substrate reacted at a similar rate in the presence of a limiting amount of purified enzyme. This sensitive assay method was used to show the virtual absence of the enzyme in brain homogenates of both male and female rats of several strains. The specific activity of whole homogenate was 0–4 extinction units/100 sec./g. of brain (six experiments).

Assays coupled to lactic dehydrogenase. Lactic dehydrogenase, phosphopyruvate kinase, enolase and phosphoglyceric acid mutase were assayed by the oxidation of DPNH accompanying conversion of pyruvate into lactate. There was little likelihood that the reversal of glycolysis would cause interference by means of the phosphoglyceraldehyde dehydrogenase reaction since this would require ATP, and these media contain ADP in three cases and none contain ATP.

(*i*) Assay of lactic dehydrogenase. The method is that described by Kornberg (1955). The 3 ml. reaction medium contained (in final concentration and at pH 7·4) triethanolamine or ammonium phosphate buffer (50 mM), sodium pyruvate (0·33 mM), DPNH (80 μM) and tissue. Rates of change of extinction up to 0·10/100 sec. were linear with respect to tissue concentration. Occasionally, in phosphate buffer, a distinct decrease in rate occurred after 3–4 min.: this was not a reproducible phenomenon and was not observed in triethanolamine buffer. Control rates were zero. About 50% loss of activity in the concentrated homogenate had occurred after 48 hr. at 2°. The specific activity of whole homogenate was 173 ± 1 extinction units/100 sec./g. of brain (four experiments).

(*ii*) Assay of phosphopyruvate kinase. The method is that described by Bücher & Pfleiderer (1955). The 3 ml. reaction medium contained (in final concentration and at pH 7·4) triethanolamine buffer (50 mM), KCl (75 mM), $MgCl_2$ (8 mM), ADP (0·23 mM), potassium phosphopyruvate (0·8 mM), DPNH (80 μM), lactic dehydrogenase coupling enzyme, and tissue. Phosphate buffer was unsuitable because of the insolubility of magnesium phosphate, and in collidine buffer the reaction rate was low, erratic and not proportional to the concentration of enzyme. Rates of change of extinction up to 0·12/100 sec. which were linear with respect to tissue concentration could be obtained with triethanolamine buffer, and control rates were negligible. The concentrated enzyme was stable in sucrose homogenates for at least 24 hr. at 2°. The specific activity of whole homogenate was 57 ± 4 extinction units/100 sec./g. of brain (two experiments).

(*iii*) Assay of enolase. The only published spectrophotometric assays depend on the absorption of light at 240 mμ by phosphopyruvate, and are not suited to crude tissue preparations, nor to more than brief reaction periods. A suitable method was devised with the same medium as for phosphopyruvate kinase, but with DL-2-phosphoglyceric acid (0·7 mM, D-isomer) as substrate: phosphopyruvate kinase and lactic dehydrogenase were used as coupling enzymes. Rates of change of extinction up to 0·12/100 sec. were linear with respect to tissue concentration, and the control rates were zero. The specific activity of whole homogenate was 50 ± 5 extinction units/100 sec./g. of brain (two experiments).

(*iv*) Assay of phosphoglyceric acid mutase. The principle of the method was outlined by Sutherland, Posternak & Cori (1949). The present method used the same medium as for phosphopyruvate kinase, but with potassium 3-phosphoglycerate (7 mM) as substrate, potassium 2:3-diphosphoglycerate (10 μM) as cofactor, and enolase, phosphopyruvate kinase and lactic dehydrogenase as coupling enzymes. The cofactor was virtually free of 2-phosphoglycerate. Rates of change of extinction which were linear with respect to tissue concentration could be obtained only up to 0·07/100 sec., but this may have been due to the sparing use of coupling enzymes. The control rates were zero. The specific activity of whole homogenate was 120 ± 3 extinction units/100 sec./g. of brain (two experiments).

Assays coupled to glyceraldehyde 3-phosphate dehydrogenase. 3-Phosphoglyceric acid kinase and glyceraldehyde phosphate dehydrogenase are assayed by causing a reversal of glycolysis, and measuring the oxidation of DPNH accompanying the conversion of 1:3-diphosphoglyceric acid into glyceraldehyde 3-phosphate. This reaction is less subject to interference from other enzymes in crude tissue preparations than is the forward reaction. In the dehydrogenase assay, one coupling enzyme is involved before the reaction step being studied: 1:3-diphosphoglyceric acid is generated *in situ*.

(*i*) Assay of glyceraldehyde phosphate dehydrogenase. The method is essentially that of Beisenherz *et al.* (1953), as improved by Wu & Racker (1959), except that triethanolamine buffer was used, and EDTA was included to prevent inactivation of the enzyme by heavy-metal impurities. The 3 ml. reaction sample contained (in final concentration, and at pH 7·4) triethanolamine buffer (50 mM), $MgCl_2$ (10 mM), EDTA (3 mM), ATP (3 mM), potassium 3-phosphoglycerate (5 mM), DPNH (80 μM), and phosphoglyceric acid kinase coupling enzyme, and tissue. A moderate rate of DPNH oxidation was observed in a preliminary control containing substrate and tissue but without coupling enzymes (see Methods). This was eliminated by 3 mM-potassium iodoacetate, and not inhibited by 0·3 mM-potassium oxalate [a lactic dehydrogenase inhibitor (Neilands, 1954)]. The

preliminary control rate was therefore not subtracted from the overall rates of reaction measured, as it appeared to be due to the reaction to be studied proceeding slowly with the aid of endogenous coupling enzyme. The method was very sensitive, but addition of α-glycerophosphate dehydrogenase and triose phosphate isomerase would increase the observed rates if greater sensitivity still were desired. Endogenous α-glycerophosphate dehydrogenase in most tissue samples other than brain would cause a variable interference best eliminated by adding a definite excess of purified enzyme; the necessity does not arise with brain, which has very small amounts of this enzyme. Tissue suspensions were diluted with 0·3M-sucrose containing 2 mM-EDTA. Rates of change of extinction up to 0·10/100 sec. were linear with respect to tissue concentration, but a definite decrease in rate occurred after 3–4 min., the slower rate being then maintained until the DPNH was consumed; the faster rate was chosen as significant. Control rates were zero. Even at very high dilutions the enzyme was stable in the presence of EDTA at 0° for at least 1 hr. The specific activity of whole homogenate was 241±4 extinction units/100 sec./g. of brain (two experiments).

(*ii*) Assay of 3-phosphoglyceric acid kinase. The method is identical with that for glyceraldehyde phosphate dehydrogenase, except that glyceraldehyde phosphate dehydrogenase is used as the coupling enzyme instead of phosphoglyceric acid kinase. Rates of change of extinction up to 0·10/100 sec. were linear with respect to tissue concentration, and the control rates were zero. The specific activity of whole homogenate was 200±8 extinction units/100 sec./g. of brain (two experiments).

Other assays

Chemical assay of hexokinase. This was based on the original method of Crane & Sols (1953, 1954*b*), with 2-deoxyglucose as substrate in a phosphate buffer. A number of variations were studied in order to obtain rates linear with respect to tissue concentration and to time for 30 min.; high ATP concentrations (10 mM) were desirable, and, although triethanolamine was not inhibitory in the presence of phosphate, reaction did not occur in triethanolamine buffer alone; other buffers were not tried. Tissue suspensions (0·2 ml.) containing EDTA (2 mM) were assayed in a medium (final vol. 1·0 ml.) containing potassium phosphate (20 mM), $MgCl_2$ (8 mM), KF (10 mM), EDTA (2 mM), 2-deoxyglucose (4 mM) and ATP (10 mM). Best results were obtained at pH 6·5–7·0: activity was high at pH 7·4, but results were more erratic, possibly owing to the decreased stability at this pH (Crane & Sols, 1954*a*). Incubation was for 30 min. at 30°, and the reaction was stopped by adding 1 ml. of 0·3N-$Ba(OH)_2$, followed by 1 ml. of 5% $ZnSO_4$ soln. The volume was made up to 5 ml. and, after centrifuging, a 2 ml. sample of the clear supernatant was analysed for reducing sugar with the alkaline Shaffer–Somogyi reagent 60 (1933) containing smaller quantities of tartrate as recommended by Somogyi (1952) for use with the arsenomolybdate reagent of Nelson (1944); determinations were made in triplicate and zero-time controls were run. The specific activity of whole homogenate was 51±4 mg. of 2-deoxyglucose consumed/30 min./g. of brain (eleven experiments).

Assay of cholinesterase. The manometric method was that described by Aldridge & Johnson (1959), in a medium containing (in final concentration) NaCl (0·13M), $MgCl_2$ (35 mM), $NaHCO_3$ (31 mM) and equilibrated at 37° with CO_2+N_2 (5:95). True cholinesterase and pseudocholinesterase were determined from the ratio of the rates of hydrolysis of acetylcholine (14 mM) and *n*-butyrylcholine (30 mM) by using the substrate ratios for the enzymes of rat brain determined by Davison (1953). With acetylcholine as substrate, the specific activity of whole homogenate was 11 700±1200 μl. of CO_2/hr./g. of brain (seven experiments).

Assay of esterase. The manometric method was the modified one described by Aldridge & Johnson (1959). The medium contained (in final concentration) NaCl (0·13M), $MgCl_2$ (35 mM), $NaHCO_3$ (31 mM), bovine-serum albumin (1·5%, w/v) and phenyl butyrate (23 μl./flask; final volume 4 ml.), equilibrated at 37° with CO_2+N_2 (5:95). The specific activity of whole homogenate was 20 000±1750 μl. of CO_2/hr./g. of brain (three experiments).

Assay of succinic dehydrogenase. The manometric method was that of Aldridge & Johnson (1959). Suspensions of ruptured particles in hypotonic solution were assayed at 37° and pH 7·5 in a medium containing (in final concentration) potassium phosphate (12·5 mM), $MgCl_2$ (3·5 mM), EDTA (0·6 mM), potassium succinate (22·5 mM), $NaHCO_3$ (25 mM) and $K_3Fe(CN)_6$ (12·5 mM), equilibrated with CO_2+N_2 (5:95). The specific activity of whole homogenate was 11 500±900 μl. of Co_2/hr./g. of brain (four experiments).

Determination of protein. The biuret procedure was used as described by Aldridge & Johnson (1959).

RESULTS

Distribution of glycolytic enzymes among particulate and supernatant fractions

When a whole brain homogenate is crudely separated into a washed particles fraction (20.particles.30) and an almost clear supernatant, the distribution of glycolytic enzymes is as shown in Table 1: hexokinase is very different from all the rest which are predominately in the supernatant fraction. So little DPN-dependent α-glycerophosphate dehydrogenase could be detected, even after freezing and thawing of samples and addition of $MgCl_2$ (40 mM) to the assay medium, that the results are hardly significant; little activity was found in brains from adult female rats of two strains, males of a third strain, and in brains from young females. The distribution of three enzymes after more complete fractionation is shown in Table 2. Most of the lactic dehydrogenase and phosphofructokinase associated with particles was found in the mitochondrial fractions. Although about 75% of the hexokinase was in the mitochondrial fractions, about 15% could not be sedimented by forces of 145 000 *g* for 60 min. No more hexokinase could be obtained in solution after freezing and thawing the mitochondria and resedimenting the particles.

Subfractionation of mitochondrial preparations

Previous work (Aldridge & Johnson, 1959) has shown that a typical (13.M.15) brain mitochondrial preparation contains about 80% of whole-homogenate succinic-dehydrogenase activity, 40% of

cholinesterase and 35% of esterase. It was believed that the latter two enzymes were associated with non-mitochondrial membranous material. Table 2 shows that about 20% of the phosphofructokinase and lactic dehydrogenase and 75% of the hexokinase are also present in such a preparation, and it was of interest to determine the extent of association of these enzymes with each other.

Fractionation in 0·3M-*sucrose.* Previous work (Aldridge & Johnson, 1959) showed that the cholinesterase and esterase of a 13.M.15 preparation were much less readily sedimented than was the succinic dehydrogenase. It has now been shown that, when a 13.M.15 fraction is subfractionated by centrifuging in 0·3M-sucrose at 2500 *g* for 15 min., hexokinase and succinic dehydrogenase are not separated significantly, the amount of sedimented and unsedimented enzymes being 54 and 46% for both; recovery was 103% for hexokinase and 94% for succinic dehydrogenase.

Fractionation in dense media. Whichever procedure was used to subfractionate the cream-coloured mitochondrial suspension according to density, two obviously distinct sets of particles were obtained: the light layer was almost white, and the heavy pellet was mainly tan-coloured, being covered by more or less of the white material according to the conditions of separation. The extent of separation differed, not only according to the density of the medium, but also according to its nature and whether procedure A or B was used. Thus with procedure A most of the white material was retained in the upper layer when a sucrose lower-layer concentration was 40% (w/v) (*d*, 1·14 g./ml.): in this case the cholinesterase content of the top layer was 80% and of the bottom layer 7% (two experiments). The same effect could be obtained with a medium of Ficoll (17–20%, w/v) in 0·3M-sucrose (*d*, 1·08 g./ml.), the cholinesterase distribution being 84–94% at the top and 3–4% at

Table 1. *Distribution of glycolytic enzymes between washed particulate* (20.*particles*.30), *and supernatant fractions of rat-brain homogenates*

Fraction designations and assay conditions were described under Methods. Assays were performed in duplicate, except for those of hexokinase which were in triplicate. Mean values are stated ±deviation for two experiments, and ±standard deviation for more than 2.

Enzyme	No. of expts.	Percentage of total recovered activity in fraction		Percentage of homogenate activity recovered
		(20.particles.30)	Supernatant	
Hexokinase	3	84±1	16±1	95±10
Phosphoglucose isomerase	1	14	86	115
Phosphofructokinase	2	24±4	76±4	77± 9
Aldolase	2	28±3	72±3	101± 1
Triose phosphate isomerase	2	15±7	85±7	91±12
α-Glycerophosphate dehydrogenase (DPN-dependent)	6	Very low activity mainly in supernatant		
Glyceraldehyde phosphate dehydrogenase	2	18±1	82±1	92± 7
Phosphoglyceric acid kinase	2	18±1	82±1	100± 3
Phosphoglyceric acid mutase	2	7±0	93±0	105± 3
Enolase	2	8±1	92±1	106± 3
Phosphopyruvate kinase	2	25±1	75±1	111± 4
Lactic dehydrogenase	3	23±1	77±1	94± 4

Table 2. *Detailed distribution of glycolytic enzymes among washed fractions of rat-brain homogenates*

Fraction designations and assay conditions were as described under Methods. Assays were performed in duplicate, except for hexokinase which were in triplicate. The poor recoveries for phosphofructokinase are due to the instability of fractions, and results are of low accuracy.

Enzyme	Total recovered activity in fraction (%)					Percentage of homogenate activity recovered in fractions
	1.N.10	4.M_1.15	13.M_2.15	145.P.60	S	
Hexokinase	9	76		15		87
			83		17	93
Phosphofructokinase	5	14	6	3	72	145
	3	11	7	4	75	68
Lactic dehydrogenase	3	22		5	70	93

the bottom (two experiments). This difference in densities required to achieve similar distribution did not appear to be due to failure to reach equilibrium within the hour of centrifuging since extending the time did not alter the effect. It seems that the effective density of the particles was drastically increased by contact with a high concentration of sucrose (40%, w/v; 1·2M).

When 0·3M-sucrose in D_2O–H_2O (70:30, v/v) mixture was used for one experiment under procedure B, the density was 1·11 g./ml. to allow for some D_2O exchange into the particles. The allowance was not sufficient and all the particles were sedimented. In the view of Beaufay *et al.* (1959) this may indicate damage of the particle membranes which renders them more permeable and subject to water-exchange; also, the oxidative phosphorylation systems in isolated brain mitochondria, prepared by the present homogenizing technique, are less stable than in liver mitochondria (Aldridge, 1957).

When 20% Ficoll in 0·3M-sucrose was used in procedure A, only 3% of the cholinesterase was sedimented, whereas in procedure B about 20% was sedimented; this was believed to indicate hindrance of transit of particles through the layer accumulating at the interface in procedure A.

The results of six fractionations in different media with procedure B are shown in Table 3. An unsatisfactory aspect is the tendency to obtain excessively high recovery values for succinic dehydrogenase; it was difficult to measure the full activity of the original particles suspended in Ficoll, and repeated freezing and thawing were necessary. A small separation of hexokinase and succinic dehydrogenase occurred in Expts. 2 and 3, but not in the rest. Attempts to obtain separation at higher densities were hindered by the difficulty of handling the more viscous medium. Lactic dehydrogenase, cholinesterase and esterase were clearly associated with the light fraction, in contrast with hexokinase and succinic dehydrogenase, which were mainly in the heavy fraction. A small separation of cholinesterase and esterase was achieved by a double fractionation by using procedure A; a 20.particles.30 preparation was fractionated by using 6% Ficoll in 0·3M-sucrose, and the heavy fraction obtained was refractionated by using 10% Ficoll in 0·3M-sucrose. The distribution in the light, medium and heavy fractions respectively was, for esterase, 22, 17 and 61%, and, for true cholinesterase, 36, 21 and 43%. Although the activity was low and the measurements were not accurate, the pseudocholinesterase appeared to be mainly in the heaviest fraction.

Table 3. *Distribution of enzyme activity between light and heavy subfractions of rat-brain mitochondrial preparations*

A 13.M.15 preparation was subfractionated by procedure B in 0·3M-sucrose media containing additions as noted. F, Ficoll (%, w/v); D_2O (%, v/v). Cholinesterase results are not corrected for the small pseudocholinesterase content.

Expt. no.	1	2	3	4	5*	6
Additions to 0·3M-sucrose	15% F	20% F	20% F	18–20% F	70% D_2O	20–22% F
	Percentage of total recovered activity in light fraction					
Hexokinase	20	54	54	31	20	33
Succinic dehydrogenase	14	32	39	26	19	31
Cholinesterase	72	75	81	81	75	69
Esterase	—	70	74	78	69	—
Lactic dehydrogenase	—	—	—	—	—	74
Protein	—	70	77	75	61	68
	Percentage of total recovered activity in heavy fraction					
Hexokinase	80	46	46	69	80	67
Succinic dehydrogenase	86	68	61	74	81	69
Cholinesterase	28	25	19	19	25	31
Esterase	—	30	26	22	31	—
Lactic dehydrogenase	—	—	—	—	—	26
Protein	—	30	23	25	39	32
	Percentage of 13.M.15 activity recovered in fractions					
Hexokinase	100	87	104	101	100	113
Succinic dehydrogenase	94	118	125	92	113	126
Cholinesterase	107	111	103	102	100	95
Esterase	—	93	94	96	95	—
Lactic dehydrogenase	—	—	—	—	—	101
Protein	—	93	94	101	92	97

* All the particles sedimented in this experiment; the upper layer of the packed pellet was removed and treated as a light layer.

DISCUSSION

The whole-homogenate fractionation experiments reveal a sharp difference between the location of hexokinase and the rest of the glycolytic enzymes (Table 1). A possible association of particulate hexokinase with that portion (about 20%) of the other glycolytic enzymes which were found in mitochondrial preparations is unlikely, for simple subfractionation of such preparations into light and heavy portions shows a great divergence of hexokinase and lactic dehydrogenase (Table 3): the latter is taken as typical of the remaining glycolytic enzymes. The fact that hexokinase could not be separated significantly from succinic dehydrogenase does not entirely prove identity of location, but is highly suggestive. To apply more extensive separative methods is not easy as brain hexokinase is comparatively unstable. In the experiment with heavy water the two enzymes remained together, indicating a similar response of their particulate 'hosts' to water exchange. The separation of lactic dehydrogenase from hexokinase and succinic dehydrogenase, and its association with cholinesterase, suggest that lactic dehydrogenase may be associated with nerve fragments, and it seems reasonable to suppose that at least some of the particulate lactic dehydrogenase represents cytoplasm entrapped in axons which have not been completely broken. A few histological studies of the subfractions show the presence of many small silver-staining nerve fibres in the light fraction and fewer coarser fragments in the heavy fraction. The fact that about 80% of the mitochondria, as measured by succinic dehydrogenase, appear to be in the heavy fraction containing only 40% of the protein indicates that there must be much non-mitochondrial material present in the original 13.M.15 pellet, and other histological studies confirm that this is so. The presence of lysosomes and other granules has been reported in brain mitochondrial preparations (Beaufay, Berleur & Doyen, 1957; Walaszek & Abood, 1957; Hebb & Whittaker, 1958; Whittaker, 1958). The gross contamination of brain mitochondrial preparations by other particles has also been shown in electron micrographs of fractions prepared in 0·88 M-sucrose (Petrushka & Giuditta, 1959).

These subfractionation studies suggest that the reports of glycolysis in brain mitochondria (Du Buy & Hesselbach, 1956; Gallagher *et al.* 1956; Balázs & Richter, 1958; Abood *et al.* 1959) reflect the limitations of the separative techniques rather than realities *in vivo*. It should also be realized that in any glycolytic studies the hexokinase content of brain mitochondrial preparations relative to other glycolytic enzymes is about four times that of whole-brain homogenate, whereas clear supernatant has a relative hexokinase content of approximately one-fifth of that of whole homogenate: this tends to vitiate attempts to elucidate glycolytic rate-controlling steps of physiological significance. M. K. Johnson (unpublished work) has found that the glycolytic activity of clear supernatant from rat-brain homogenate is about one-third of that of a whole homogenate, and is trebled by addition of only 60% of the equivalent brain mitochondria. This is in accord with the work of Rossiter (1957). The low rate presumably reflects the activity of the small amount of soluble hexokinase in the clear supernatant: addition of brain mitochondria makes good the deficiency of hexokinase. Whether the soluble hexokinase is a physiological reality or an artifact of homogenization is not clear. Attempts to liberate any more hexokinase from the mitochondria by freezing and thawing were not successful. Crane & Sols (1953) failed to liberate hexokinase into solution by homogenizing brain in water or potassium phosphate buffer instead of sucrose. It is tempting to speculate that the soluble hexokinase is involved in basal metabolism of glucose and that the higher rates of glycolysis required for active metabolism are controlled in some way by access to the soluble enzymes of glucose 6-phosphate formed in the mitochondria.

It is difficult to attach much significance to the variation of content of the mainly soluble glycolytic enzymes in the particulate fraction; this was as low as 8% for phosphoglyceric acid kinase and enolase, and as high as 28% for aldolase. In one experiment in which brain was homogenized by using a Nelco blade homogenizer, the particles contained only 10% of the lactic dehydrogenase instead of 23%. This supports the view that the particulate enzyme is entrapped within poorly broken fragments rather than adsorbed on the surfaces.

The absence or very low activity of the DPN-dependent α-glycerophosphate dehydrogenase is in accord with the report by Green, Needham & Dewan (1937) that such an enzyme could not be found in ox brain, but in disagreement with Boxer & Shonk (1960), who report levels in rat brain about one-quarter of that of the lactic dehydrogenase activity. These workers used a similar assay procedure but give insufficient details for a comparison with the present work. I found no large change according to age, sex or breed of rat, nor was purified muscle α-glycerophosphate dehydrogenase inactivated when diluted with cold brain homogenate and stored for 2 hr. at 0°. Green (1936) also reported the presence of an active particulate non-DPN-dependent α-glycerophosphate dehydrogenase in rat brain, and this has been shown to be associated with mitochondrial particles (Ringler & Singer, 1958).

SUMMARY

1. Homogenates of rat brain have been fractionated by centrifuging in 0·3 M-sucrose, and mitochondrial preparations have been subfractionated in dense media.

2. Part (15%) of the hexokinase could not be sedimented, but 75% was present in the mitochondrial fractions and could not be separated from succinic dehydrogenase. The hexokinase in the mitochondrial fraction was not liberated by freezing and thawing.

3. Each of the other glycolytic enzymes was present mainly (70–90%) in the clear cytoplasmic fraction.

4. Only traces of DPN-dependent α-glycerophosphate dehydrogenase could be detected in brain homogenates.

5. The particulate portions having lactic-dehydrogenase and phosphofructokinase activity were mainly associated with the mitochondrial fraction.

6. Substantial separation of the cholinesterase, esterase and lactic dehydrogenase from the succinic dehydrogenase and hexokinase of brain-mitochondrial preparations was achieved: a light fraction containing 70–80% of the former three enzymes and a heavy fraction containing 70–80% of the latter two enzymes could be obtained.

7. A slight separation of cholinesterase and esterase was achieved by a double subfractionation of brain particles.

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REFERENCES

Abood, L. G., Brunngraber, E. & Taylor, M. (1959). *J. biol. Chem.* **234**, 1307.

Aldridge, W. N. (1953). *Biochem. J.* **53**, 62.

Aldridge, W. N. (1957). *Biochem. J.* **67**, 423.

Aldridge, W. N. & Johnson, M. K. (1959). *Biochem. J.* **73**, 270.

Balázs, R. & Richter, D. (1958). *Biochem. J.* **68**, 5P.

Baranowski, T. & Niederland, T. R. (1949). *J. biol. Chem.* **180**, 543.

Beaufay, H., Bendall, D. S., Baudhin, P., Wattiaux, R. & de Duve, C. (1959). *Biochem. J.* **73**, 628.

Beaufay, H., Berleur, A. M. & Doyen, A. (1957). *Biochem. J.* **66**, 32P.

Beisenherz, G. (1955). In *Methods in Enzymology*, vol. 1, p. 387. New York: Academic Press Inc.

Beisenherz, G., Boltze, H. J., Bücher, T., Czok, R., Garbade, K. H., Meyer-Arendt, E. & Pfleiderer, G. (1953). *Z. Naturf.* **8B**, 555.

Beisenherz, G., Bücher, T. & Garbade, K. H. (1955). In *Methods in Enzymology*, vol. 1, p. 391. New York: Academic Press Inc.

Boxer, G. E. & Shonk, C. E. (1960). *Cancer Res.* **20**, 85.

Bücher, T. & Pfleiderer, G. (1955). In *Methods in Enzymology*, vol. 1, p. 435. New York: Academic Press Inc.

Crane, R. K. & Sols, A. (1953). *J. biol. Chem.* **203**, 273.

Crane, R. K. & Sols, A. (1954*a*). *J. biol. Chem.* **206**, 925.

Crane, R. K. & Sols, A. (1954*b*). *J. biol. Chem.* **210**, 581.

Davison, A. N. (1953). *Biochem. J.* **54**, 583.

Du Buy, H. G. & Hesselbach, M. L. (1956). *J. Histochem. Cytochem.* **4**, 363.

Gallagher, C. H., Judah, J. D. & Rees, K. R. (1956). *Biochem. J.* **62**, 436.

Green, D. E. (1936). *Biochem. J.* **30**, 629.

Green, D. E., Needham, D. M. & Dewan, J. G. (1937). *Biochem. J.* **31**, 2327.

Hebb, C. O. & Whittaker, V. P. (1958). *J. Physiol.* **142**, 187.

Hers, H. G., Berthet, J., Berthet, L. & de Duve, C. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 21.

Holter, H. & Moller, K. M. (1958). *Exp. Cell Res.* **15**, 631.

Kornberg, A. (1955). In *Methods in Enzymology*, vol. 1, p. 441. New York: Academic Press Inc.

Kuff, E. L. & Schneider, W. C. (1954). *J. biol. Chem.* **206**, 677.

Long, C. (1952). *Biochem. J.* **50**, 407.

Mateyko, G. M. & Kopac, M. J. (1959). *Exp. Cell Res.* **17**, 524.

Neilands, J. B. (1954). *J. biol. Chem.* **208**, 225.

Nelson, N. (1944). *J. biol. Chem.* **153**, 375.

Petrushka, E. & Giuditta, A. (1959). *J. biophys. biochem. Cytol.* **6**, 129.

Racker, E. (1947). *J. biol. Chem.* **167**, 843.

Ringler, R. L. & Singer, T. P. (1958). *Biochim. biophys. Acta*, **29**, 661.

Rossiter, R. J. (1957). *Canad. J. Biochem. Physiol.* **35**, 579.

Shaffer, P. A. & Somogyi, M. (1933). *J. biol. Chem.* **100**, 695.

Slein, M. W. (1955). In *Methods in Enzymology*, vol. 1, p. 304. New York: Academic Press. Inc.

Somogyi, M. (1952). *J. biol. Chem.* **195**, 19.

Sutherland, E. W., Posternak, T. & Cori, C. F. (1949). *J. biol. Chem.* **181**, 153.

Thomson, J. F. & Klipfel, F. J. (1958). *Exp. Cell Res.* **14**, 612.

Walaszek, E. J. & Abood, L. G. (1957). *Fed. Proc.* **16**, 133.

Whittaker, V. P. (1958). *Biochem. Pharmacol.* **1**, 351.

Wu, R. & Racker, E. (1959). *J. biol. Chem.* **234**, 1029.

The Purification and Properties of the Tyrosine–2-Oxoglutarate Transaminase of *Saccharomyces cerevisiae*

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Studies by SentheShanmuganathan (1956, 1960) and SentheShanmuganathan & Elsden (1958) have established that in the production of fusel oil by *Saccharomyces cerevisiae* transamination is the first step in the conversion of an amino acid into the corresponding alcohol containing one less carbon atom. The formation of tyrosol by washed-cell suspensions and by cell-free extracts of yeast has been studied in detail (SentheShanmuganathan & Elsden, 1958). The work described in this paper deals with the purification and resolution of the tyrosine–2-oxoglutarate transaminase from *S. cerevisiae* and the subsequent studies of kinetics and substrate specificity with the purified enzyme. A preliminary report of these findings has been published (SentheShanmuganathan, 1958).

MATERIALS AND METHODS

Organism. The organism used throughout this work was *Saccharomyces cerevisiae* (strain 5 of the Departmental collection). The method of growing the cells and the preparation of washed cells have been described by SentheShanmuganathan & Elsden (1958).

Amino acids. All the amino acids (L. Light and Co. Ltd., Colnbrook, Bucks.) were checked for purity by paper chromatography (Woiwod, 1949). Solutions of the amino acids were adjusted to pH 7·2 with 0·2N-NaOH before use.

Phosphate buffers. The phosphate buffers used in this work were Na_2HPO_4–KH_2PO_4 mixtures.

2-Oxo acids. 2-Oxoglutarate was prepared by the method of Clutterbuck (1927). Oxaloacetic acid was a gift from Professor Sir Hans Krebs, F.R.S.; the sodium salt was prepared as described by Elsden & Ormerod (1956). *p*-Hydroxyphenyl pyruvate was prepared as described by SentheShanmuganathan & Elsden (1958).

Pyridoxal phosphate (synthetic). This was the gift of Dr Morrison of Roche Products Ltd., Welwyn Garden City, Herts.

Preparation of extracts to test transaminase activity. Dialysed cell-free extracts (termed 'Hughes extracts') of *S. cerevisiae* were used in all the experiments. The mass of broken cells, obtained by crushing the yeast in a Hughes press (Hughes, 1951), was incubated with 0·1M-phosphate buffer, pH 7·2 (12 ml./g. dry wt. of cells), for 1 hr. at 37°. The mixture was then centrifuged at 19 600 *g* for 20 min. at −2°, the supernatant was separated and the cell debris suspended in an equal volume of the same buffer. The pH values of extracts (i.e. the supernatants) so obtained varied between 6·5 and 6·7 and were adjusted to pH 7·2 with 0·2N-NaOH. The extracts contained a high concentration of free glutamate and this was eliminated by dialysing against 0·02M-KCl at 1–2° for 16–18 hr. with continuous stirring (Bigger-Gehring, 1955). The contents of the dialysing bag did not contain any precipitate. Both the dialysed extract and the suspension of cell debris were tested for transaminase activity between 2-oxoglutarate and either tyrosine or leucine. The whole of the transaminase activity was found in the cell-free extract.

Estimation of protein. This was by the turbidimetric method of Bücher (1947) adapted by Kornberg (1950) for use with the spectrophotometer. The details of the procedure used in this Department are as follows. To the sample containing no more than 0·8 mg. of protein in 2·6 ml. is added 0·3 ml. of 0·6M-$(NH_4)_2SO_4$ and *E* at 340 mμ is measured with the Unicam SP. 500 spectrophotometer and a cuvette of 1 cm. light-path. Trichloroacetic acid (0·1 ml. of 50%, w/v) is then added, the whole mixed and *E* is measured again after 2 min. With most proteins, including those in microbial extracts, the increase in extinction is directly proportional to the amount of protein present; this, however, does not hold for serum proteins (Dr J. L. Peel, unpublished work). The slope of the calibration curve varied somewhat with the protein used as standard, but approximated to an increase in extinction of 1·0/mg. of protein. Since the choice of the protein standard is arbitrary this value was, for convenience, used for all determinations.

Estimation of glutamic acid. Glutamic acid was estimated by the specific decarboxylase method of Gale (1945). The formation of glutamate was also followed by paper-partition chromatography with propanol–water (80:20). This solvent was chosen because it gave a good separation of glutamate from glutathione (Hanes, Hird & Isherwood, 1952). After the chromatogram had been developed, glutamic acid was detected by spraying with a solution of ninhydrin (Woiwod, 1949).

Assay of transaminase activity. Incubations were made in duplicate in 15 ml. test tubes at 37° unless stated to the contrary. Except where indicated differently, the complete system contained, in a total volume of 3 ml.: pyridoxal phosphate (0·04 μmole), enzyme solution (0·1 ml.), 2-oxoglutarate (10 μmoles when tyrosine was the amino acid and 20 μmoles in all other cases) and glutathione (1 μmole) in 67 mM-phosphate buffer; during the purification procedure, the pH value was 7·2 and in studies with the purified enzyme the pH value was 7·8. The mixture was incubated for 10 min. before the addition of L-tyrosine (5 μmoles) or L-leucine (10 μmoles). Two control systems

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were used, one without the amino acid and the other without 2-oxoglutarate. At the end of the incubation period, 0·2 ml. of 40% (w/v) trichloroacetic acid was added and, after heating in a boiling-water bath for 3 min., the denatured protein was removed by centrifuging. Glutamate or *p*-hydroxyphenyl pyruvate was estimated in the supernatant. No glutamate or *p*-hydroxyphenyl pyruvate was formed in the control systems. One unit of enzyme activity is defined as that amount of enzyme which catalyses the formation of 1 μmole of *p*-hydroxyphenyl pyruvate or glutamate in 30 min. at 37° under the standard conditions described above. The specific activity is the number of units of activity/mg. of protein and the purification factor is the ratio of the specific activity of the purified fraction to that of the initial material.

Estimation of 2-oxo acids. 2-Oxoglutarate was estimated by the method of Krebs (1950). *p*-Hydroxyphenyl pyruvate was estimated by the method of Briggs (1922) as modified by Canellakis & Cohen (1956). The extinction of the coloured solution obtained by mixing the *p*-hydroxyphenyl pyruvate with a mixture of ammonium phosphomolybdate and KH_2PO_4 was determined, after 1 hr., with the EEL colorimeter (Evans Electroselenium Ltd., Harlow, Essex) and a red filter. With this 2-oxo acid, a linear relationship was obtained between colour intensity and concentration up to 0·55 μmole/ml. (Fig. 1).

RESULTS

Thiol groups and activity

In the early experiments there was a rapid loss of transaminase activity during purification of the enzyme. Activity was restored by the addition of glutathione or cysteine, indicating that SH groups were probably important for optimum activity of the transaminase. Because of these results, *p*-chloromercuribenzoate, a reagent known to combine with SH groups, was tested for its effect on transaminase activity in 'Hughes extracts'. 2·83 μM-*p*-Chloromercuribenzoate inhibited transaminase activity by 56%, but increasing the concentration to as much as 0·565 mM did not cause any appreciable increase in the degree of inhibition (Table 1). In these experiments the *p*-chloromercuribenzoate was incubated together with the enzyme and pyridoxal phosphate for 10 min. before the addition of the tyrosine. Inhibition may have been only partial because of protection of some of the SH groups by the cofactor. However, if the cofactor was added after *p*-chloromercuribenzoate had been incubated with the enzyme for 10 min., the results were essentially the same as before. Since these experiments were done with the crude cell-free extract it was possible that two or more transaminases for tyrosine were present and that SH groups were essential for the optimum activity of only one of the enzymes. Alternatively, if only one transaminase is present in the crude extract, it is feasible that only some of the SH groups are required for maximal activity. It is well known that in some enzymes, e.g. adenosine triphosphatase (Singer & Barron, 1944), all the thiol groups of the native protein are essential for activity, whereas in other enzymes, e.g. urease (Hellerman, Chinard & Deitz, 1943), only some of them are essential. Reactivation of transaminase inhibited by *p*-chloromercuribenzoate was achieved by the addition of glutathione (0·01 M) or cysteine (0·01 M) to the reaction mixture (Table 2). In subsequent experiments glutathione was always included in the test system for transaminase activity.

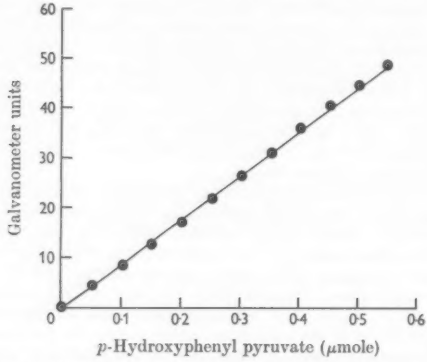

Fig. 1. Calibration curve (EEL spectrophotometer readings) for the estimation of *p*-hydroxyphenyl pyruvate. Estimated by the method of Canellakis & Cohen (1956).

Purification procedure for tyrosine transaminase

The general properties of the transaminase with regard to the stability to acid and heat and to adsorption on to calcium phosphate gel and subsequent elution were established in small-scale experiments. Table 3 summarizes the steps and

Table 1. *Effect of* p*-chloromercuribenzoate on tyrosine–2-oxoglutarate-transaminase activity*

The system (3 ml.) containing the enzyme (0·1 ml. of Hughes extract), pyridoxal phosphate (0·04 μmole), 2-oxoglutarate (10 μmoles) and *p*-chloromercuribenzoate was incubated for 10 min. before the addition of L-tyrosine (5 μmoles). *p*-Hydroxyphenyl pyruvate formed in 30 min. at 37° was estimated by the method of Canellakis & Cohen (1956). (Unit of activity is defined in the Materials and Methods section.)

Concn. of *p*-chloromercuribenzoate (μM)	Total transaminase activity (units/ml.)	Inhibition (%)
0	22·8	—
2·83	10·2	56
5·66	10·1	56
8·49	10·2	56
11·30	10·1	56
565	10·2	56

Table 2. *Reactivation of the tyrosine–2-oxoglutarate transaminase inhibited by* p-*chloromercuribenzoate*

The system (3 ml.), containing the enzyme solution (0·1 ml. of Hughes extract), pyridoxal phosphate (0·04 μmole), 2-oxoglutarate (10 μmoles) and *p*-chloromercuribenzoate (as given), was incubated for 10 min.; then either glutathione or cysteine (0·01 M) was added and the mixture incubated for a further period of 10 min. before addition of L-tyrosine (5 μmoles). *p*-Hydroxyphenyl pyruvate formed in 30 min. at 37° was estimated by the method of Canellakis & Cohen (1956).

Concn. of *p*-chloromercuribenzoate (μM)	Total transaminase activity (units/ml.)		
	No addition	Glutathione (0·01 M)	Cysteine (0·01 M)
0	22·8	31·2	31·2
2·83	10·2	31·2	31·0
5·66	10·1	30·8	30·8

yields of a typical preparation. In order to obtain sufficient material and because freshly grown yeast was required, a series of extracts was prepared over a period of 2 weeks. Each extract was dialysed against mM-phosphate buffer, pH 7·2, for 12–16 hr. at 1–2° and then stored at −20°. All the preparations were subsequently mixed to provide the initial preparation. Acid precipitation was the first step. Acetic acid (2N) was added rapidly until the pH value was 4·1; the preparation was then centrifuged at 19 600 *g* for 20 min. at −2° and the precipitate discarded (yield of enzyme: 95–97% with a sixfold purification). The supernatant was adjusted to pH 5·5 with 0·2N-NaOH and the temperature raised rapidly (4 min.) to 60°. The preparation was kept at this temperature for 6 min. and cooled and the inactive precipitate was removed by centrifuging. This treatment gave a twofold purification.

Table 3. *Purification of tyrosine transaminase*

Step	Volume (ml.)	Protein (mg.)	Total activity (units)	Specific activity	Purification factor	Yield (%)
(i) Dialysed crude cell-free extract	380	10 640	5 152	0·48	1	100
(ii) Acid treatment: brought to pH 4, ppt. discarded	360	1 400	5 010	3·5	7	97
(iii) Heat treatment. Supernatant from (ii) at 60° for 6 min. at pH 5·5, ppt. discarded	350	600	4 200	7·0	14	81
(iv) $Ca_3(PO_4)_2$ gel treatment:						
(1) 0·66 mg. (dry wt. of gel/mg. of protein)	40	140	900	6·4	13	17
(2) 0·5 mg. (dry wt. of gel/mg. of protein)	40	105	2 140	20	41	41
(3) 0·5 mg. (dry wt. of gel/mg. of protein)	30	136	1 010	7·4	15	19
Gel from 1 and 3 combined, eluted and eluate treated						
(1) 0·66 mg. (dry wt. of gel/mg. of protein)	—	—	—	—	—	—
(2) 0·5 mg. (dry wt. of gel/mg. of protein)	40	60	1 068	17	35	20
(3) 0·5 mg. (dry wt. of gel/mg. of protein)	—	—	—	—	—	—
(v) $(NH_4)_2SO_4$ fractionation of combined (iv) two eluates						
Unfractionated	100	165	3 208	19	39	62
0–50%	20	25	132	5·2	11	2
50–65%	30	80	2 329	29	60	45
65–85%	20	37	642	17	35	12
(vi) $(NH_4)_2SO_4$ fractionation of (v) 50–65%						
Unfractionated	40	80	2 344	29·3	61	45
0–50%	—	0	—	—	—	—
50–58%	15	40	1 652	41·3	86	32
58–65%	15	20	680	34	70	13
65–75%	5	6	48	8	—	—
(vii) $(NH_4)_2SO_4$ fractionation of 50–58% and 58–65% from (vi)	40	60	2 344	40	83	45
0–50%	—	0	—	—	—	—
50–54%	15	20	1 071	53	110	20
54–60%	15	14	688	49	100	13
60–65%	15	16	375	22	46	7

The supernatant resulting from the heat-treatment was brought to pH 6·0 and stirred with calcium phosphate gel (final concentration: 0·66 mg. of gel/mg. of protein). The gel (P 1), which was collected by centrifuging, contained only 17 % of the initial activity. A further 0·5 mg. of gel/mg. of protein was added and the resulting precipitate (P 2) contained 41 % of the activity. A third treatment with 0·5 mg. of gel/mg. of protein gave precipitate P 3, which contained a further 19 % of the activity. The enzyme was eluted from P 2 by stirring it with 0·1 M-phosphate buffer, pH 7·2, for 1 min. The gel was centrifuged off and the eluate stored at −20° until required. Meanwhile, P 1 and P 3 were pooled, the enzyme was eluted with phosphate buffer as described above and the eluate dialysed against mM-phosphate buffer, pH 7·2, for 12–16 hr. The dialysed eluate so obtained was then subjected to a further gel treatment, the eluate from which contained 20 % of the original activity. This was mixed with the eluate from P 2.

The combined eluates were brought to 50 % saturation with $(NH_4)_2SO_4$ and after 5 min. the precipitate was removed by centrifuging (19 600 *g* for 10 min. at −2°). The supernatant was brought to pH 7·2 (0·2 N-NaOH) and the $(NH_4)_2SO_4$ concn. to 65 % saturation; the precipitate was removed by centrifuging. The supernatant was again brought to pH 7·2 and the $(NH_4)_2SO_4$ concn. to 85 % and the precipitate was collected. Each precipitate was dissolved in 0·1 M-phosphate buffer, pH 7·2, and insoluble material was removed. The bulk of the enzyme (45 %) was in the 50–65 % fraction, with a 60-fold purification, and this fraction was refractionated between the limits 0–50, 50–58, 58–65 and 65–75 % (w/v) $(NH_4)_2SO_4$. Activity was mainly in the 50–58 % and 58–65 % fractions; these two fractions were combined and refractionated. The 50–54 % and 54–60 % fractions contained most of the activity (20 %, purification 110 times, and 13 %, purification 100 times, respectively). Dialysis of the 54–60 % fraction produced no further change and the material from this fraction was used in the subsequent studies of the properties of the tyrosine–2-oxoglutarate transaminase. This preparation had no effect on the four reactants (L-tyrosine, 2-oxoglutarate, *p*-hydroxyphenyl pyruvate and L-glutamic acid) when incubated with any one of them alone.

Properties of the partially purified tyrosine transaminase

Specificity. The specificity of the purified enzyme preparation was examined by incubating it with various amino acids, 2-oxoglutarate, glutathione and buffer at pH 7·8. At the end of the incubation period, the mixture was held at 100° for 5 min. to inactivate the enzyme and was then centrifuged. The formation of glutamic acid, detected by paper chromatography, was taken as an indication of enzymic activity. For comparative purposes equal volumes of the various supernatants were placed on the chromatograms. Of the ten amino acids tested for their ability to transaminate with 2-oxoglutarate, only tyrosine gave rise to a large distinct spot corresponding to glutamic acid. Leucine, norleucine, methionine, tryptophan and phenylalanine formed only very small amounts of glutamic acid (faint spots) and there was no detectable glutamic acid formed from isoleucine,

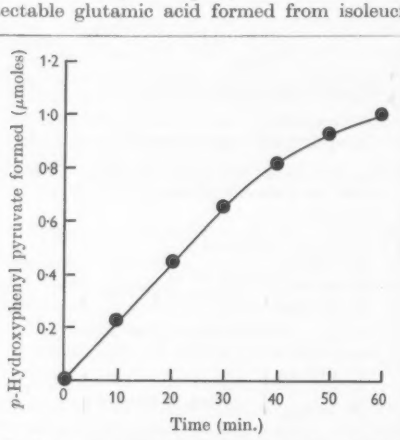

Fig. 2. Progress curve for the formation of a *p*-hydroxyphenyl pyruvate from tyrosine. Experimental conditions are described in the text. The experimental system was contained in 0·1 M phosphate buffer, pH 7·8. Temp., 37°. Amount of enzyme protein used was 6·67 μg. The keto acid formed was measured at 10 min. intervals.

Table 4. *Activities of leucine- and tyrosine-transaminase systems*

Preparation 1 was a dialysed crude extract and preparation 2 was obtained by adjusting preparation 1 to pH 4·0 and discarding the precipitate. Transaminase activity was measured as described in the text.

		Leucine transaminase		Tyrosine transaminase		
Preparation	Total protein (mg.)	Total units in extract	Specific activity (units/mg.)	Total units in extract	Specific activity (units/mg.)	Specific activity (tyrosine) / Specific activity (leucine)
1	2200	2400	1·1	1310	0·59	0·54
2	320	630	1·9	1230	4·0	2·11

valine, norvaline and aspartic acid. The dialysed crude extract catalysed the transamination of 2-oxoglutarate with each of the ten amino acids tested and glutamic acid was formed in good yield (SentheShanmuganathan, 1960); it is clear that the final preparation had been substantially freed from these other transaminases. In the dialysed crude Hughes extract, the activity for leucine was nearly twice that for tyrosine, as judged by the rate of glutamic acid formation. After acid precipitation at pH 4, only 26 % of the leucine activity remained in the supernatant; the activity for tyrosine remained unchanged (Table 4). Hence it would appear that transamination involving leucine is not catalysed by the same enzyme as that concerned with tyrosine. The ability of the purified enzyme to transfer amino groups from L-tyrosine to pyruvate or oxaloacetate was also tested, but neither of these keto acids could replace 2-oxoglutarate. The amount of *p*-hydroxyphenyl pyruvate formed by the complete system at pH 7·8 increased linearly with time up to 30 min. (Fig. 2), and in all further work the incubation period was 30 min. or less.

Effect of pH *on activity.* With the standard incubation system, but with different buffers, the effect of pH on activity was determined. The optimum pH was between pH 7·8 and 8·0 at 37° (Fig. 3). The activity fell sharply on the acid side of the optimum pH but decreased only a small amount on the alkaline side (up to pH 10).

Effect of enzyme concentration. The rate of formation of *p*-hydroxyphenyl pyruvate as a function of the amount of enzyme preparation added to the system was linear up to about 4·5 μg. of protein/ml. of reaction mixture (Fig. 4). With higher concentrations of the enzyme preparation the rate declined, which may have been due to inhibition by the *p*-hydroxyphenyl pyruvate produced in the reaction (cf. Canellakis & Cohen, 1956).

Effect of pyridoxal phosphate. The purified enzyme was completely resolved and was inactive unless pyridoxal phosphate was added to the system. The enzyme was saturated at 2·8 μM-pyridoxal phosphate (Fig. 5).

Effect of temperature. The rate of transamination increased with temperature, and there was a linear relationship between 25° and 37° (Fig. 6). The temperature coefficient (Q_{10}) over the range 27–37° was 1·87.

Michaelis constants for tyrosine, 2-oxoglutarate and coenzyme. In order to determine the Michaelis constants (K_m) for any one of the reactants tyrosine, 2-oxoglutarate and pyridoxal phosphate, sufficient of the other two compounds must be present to ensure that the enzyme system is saturated and that the only material influencing the rate of the reaction is the substance under test (cf. Alberty, 1953). Precautions were taken to ensure that these conditions applied, and the observed rates of reaction were plotted graphically according to the method of Lineweaver & Burk (1934). The K_m values obtained from the three experiments in which the concentration of tyrosine,

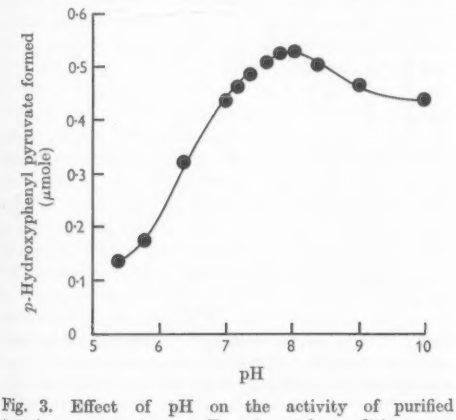

Fig. 3. Effect of pH on the activity of purified tyrosine transaminase. Experimental conditions are described in the text. Phosphate buffers (0·1 M) were used up to pH 7·5; above pH 7·5 pyrophosphate buffers (0·1 M) were used. Temp., 37°. The amount of *p*-hydroxyphenyl pyruvate formed in 30 min. was determined. The amount of enzyme protein used was 6·67 μg.

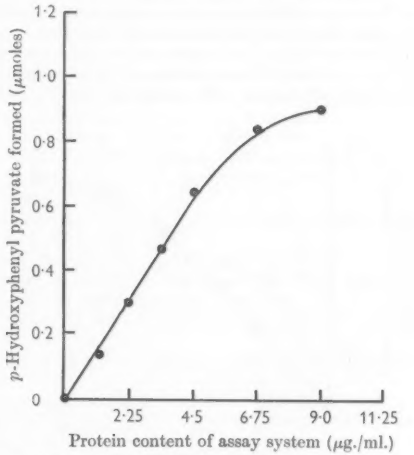

Fig. 4. Effect of enzyme concentration on the formation of *p*-hydroxyphenyl pyruvate from tyrosine. Experimental conditions are described in the text. The purified enzyme preparation used contained 66·7 μg. of protein/ml., in 0·1 M-phosphate buffer, pH 7·8. Temp., 37°; incubation period 30 min. The activity was measured in the presence of different amounts of the enzyme.

2-oxoglutarate and pyridoxal phosphate were varied one at a time are shown in Table 5, and, for comparison, those obtained by Canellakis & Cohen (1956) for the analogous enzyme from dog liver are also included.

Energy of activation and change in heat content of enzyme–substrate complex. With different concentrations of L-tyrosine and saturating concentrations of 2-oxoglutarate and pyridoxal phosphate, the maximum velocities of reaction at different temperatures were calculated from the rates of reaction according to the method of Lineweaver & Burk (1934). There was a linear relationship between $\log(V_{max.})$ and the reciprocal of the absolute temperature (Fig. 7), and since the slope of the graph is $-Ea/R$ (Gibson, 1953), Ea, the energy of activation, is 15·2 kg.cal. for the temperature range 25–46°.

If the Michaelis–Menten theory is assumed to apply, then since K_m is the reciprocal of an equilibrium constant it can be deduced from the van 't Hoff isochore that

$$\frac{d(\log K_m)}{d(1/T)} = \frac{\Delta Hm}{R},$$

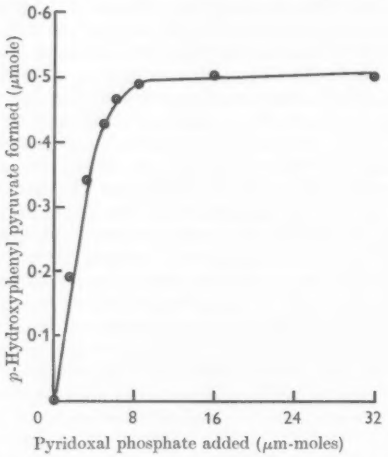

Fig. 5. Effect of concentration of pyridoxal phosphate on the formation of *p*-hydroxyphenyl pyruvate from tyrosine. Experimental conditions are given in the text. Pyridoxal phosphate was added as required. The purified enzyme (equivalent to 6·67 μg. of protein) and the substrates were present in 0·1 M-phosphate buffer, pH 7·8. *p*-Hydroxyphenyl pyruvate formed in 30 min. at 37° was estimated.

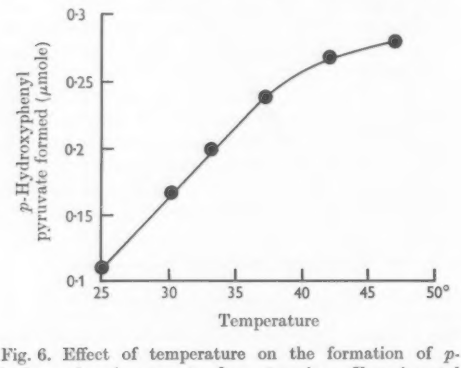

Fig. 6. Effect of temperature on the formation of *p*-hydroxyphenyl pyruvate from tyrosine. Experimental details are given in the text. The system was contained in 0·1 M-phosphate buffer, pH 7·8. The initial rate of formation of *p*-hydroxyphenyl pyruvate at each temperature was measured at the end of 10 min. incubation period.

Table 5. *K_m values for tyrosine, 2-oxoglutarate and pyridoxal phosphate*

The complete system contained in a total volume of 3·0 ml.: pyridoxal phosphate (48 μM), L-tyrosine (33 mM) and 2-oxoglutarate (50 mM) in 0·1 M-phosphate buffer, pH 7·8; Temp., 37°.

	K_m	
Substrate	Yeast enzyme	Dog-liver enzyme
Tyrosine	1·66 mM	0·71 mM
2-Oxoglutarate	2·5 mM	0·16 mM
Pyridoxal phosphate	2·38 μM	1·0 μmM

Fig. 7. Determination of energy of activation of enzyme-substrate complex. Maximum reaction velocity ($V_{max.}$) was determined at different temperatures. At each temperature the rate of reaction for different tyrosine concentrations was measured and $V_{max.}$ determined by the method of Lineweaver & Burk (1934). Slope of graph $= -Ea/R$. The experimental system contained, in a total volume of 3·0 ml. in 0·1 M-phosphate buffer, pH 7·8: pyridoxal phosphate (48 μM), 2-oxoglutarate (50 mM), enzyme solution (0·1 ml., 6·67 μg. of protein). Incubation period was 10 min.

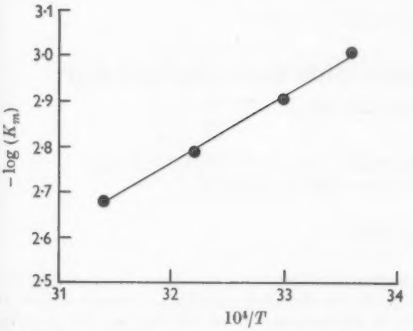

Fig. 8. Determination of change in heat-content in the formation of enzyme–substrate complex. Michaelis constant (K_m) for tyrosine was determined at different temperatures. At each temperature the rate of reaction for different tyrosine concentrations was measured and then K_m was determined by the method of Lineweaver & Burk (1934). The concentrations of the various substrates added were as described under Fig. 7.

where ΔHm is the change in the heat content accompanying the formation of the enzyme–substrate complex. Experiment showed a linear relationship between $\log K_m$ and the reciprocal of the absolute temperature (Fig. 8). The slope equalled $(30/21) \times 10^3$ and corresponds to $\Delta Hm/R$, whence ΔHm is found to be 6·5 kg.cal. for the temperature range 25–46°.

DISCUSSION

Saccharomyces cerevisiae can bring about transamination reactions involving several amino acids, and it seems likely from the work described here that there is more than one transaminase responsible for these reactions. The preparation of the tyrosine enzyme obtained, although not pure, showed little transaminase activity for amino acids other than tyrosine, whereas crude cell-free extracts contain a leucine transaminase apparently more active than the tyrosine enzyme. Like other transaminases, the tyrosine enzyme was activated by pyridoxal phosphate, and the enzyme was saturated when the coenzyme concentration was 2·8 μM. This value is close to that (4 μM) obtained by Canellakis & Cohen (1956) for the tyrosine transaminase of dog liver. No evidence has been obtained that the purified tyrosine transaminase of yeast has any properties which are markedly different from those of other purified transaminases (cf. O'Kane & Gunsalus, 1947).

SUMMARY

1. Tyrosine–2-oxoglutarate transaminase has been purified more than 100-fold with an overall yield of 33% from a dialysed crude extract of *Saccharomyces cerevisiae*.

2. The purified enzyme required pyridoxal phosphate for activity, and glutathione increased transaminase activity by 50%.

3. Neither pyruvate nor oxaloacetate replaced 2-oxoglutarate and only a very slight activity was observed when tryptophan, methionine, phenylalanine, leucine or norleucine replaced tyrosine, suggesting that the enzyme is highly specific.

4. At pH 7·8 and 37° the Michaelis constants for tyrosine, 2-oxoglutarate and pyridoxal phosphate are 1·66 mM, 2·5 mM and 2·38 μM respectively.

5. The temperature coefficient over the range 27–37° is 1·87 and the energy of activation determined over the range 25–46° is 15·2 kg.cal. The change in heat content accompanying the formation of the enzyme–substrate complex is 6·5 kg.cal. over the temperature range 25–46°.

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REFERENCES

Alberty, R. A. (1953). *J. Amer. chem. Soc.* **75**, 1928.
Bigger-Gehring, L. (1955). *J. gen. Microbiol.* **13**, 45.
Briggs, A. P. (1922). *J. biol. Chem.* **51**, 453.
Bücher, T. (1947). *Biochim. biophys. Acta*, **1**, 292.
Canellakis, Z. N. & Cohen, P. P. (1956). *J. biol. Chem.* **222**, 53.
Clutterbuck, P. W. (1927). *Biochem. J.* **21**, 512.
Elsden, S. R. & Ormerod, J. G. (1956). *Biochem. J.* **63**, 691.
Gale, E. F. (1945). *Biochem. J.* **39**, 46.
Gibson, K. D. (1953). *Biochim. biophys. Acta*, **10**, 121.
Hanes, C. S., Hird, F. J. R. & Isherwood, F. A. (1952). *Biochem. J.* **51**, 25.
Hellerman, L., Chinard, F. P. & Deitz, V. R. (1943). *J. biol. Chem.* **147**, 443.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
Kornberg, A. (1950). *J. biol. Chem.* **182**, 779.
Krebs, H. A. (1950). *Biochem. J.* **47**, 605.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
O'Kane, D. E. & Gunsalus, I. C. (1947). *J. biol. Chem.* **170**, 425.
SentheShanmuganathan, S. (1956). *Biochem. J.* **64**, 37P.
SentheShanmuganathan, S. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, no. 4–64, p. 46.
SentheShanmuganathan, S. (1960). *Biochem. J.* **74**, 568.
SentheShanmuganathan, S. & Elsden, S. R. (1958). *Biochem. J.* **69**, 210.
Singer, T. P. & Barron, E. S. G. (1944). *Proc. Soc. exp. Biol., N.Y.*, **56**, 120.
Woiwod, A. J. (1949). *Biochem. J.* **45**, 412.

Biochem. J. (1960) 77, 626

Fatty Acid Composition of Phospholipids from Subcellular Particles of Rat Liver

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The object of this work was to determine the fatty acid composition of the different phospholipids present in the mitochondria and microsomes of rat liver. Clément, Haimovici & Le Breton (1955) obtained data for the polyenoic acids of the neutral fat and of the total phospholipids, by spectrophotometry after alkali-isomerization, and Clément, Clément & Le Breton (1956) gave further data on the nature of the lipids. It appeared of interest to obtain more detailed information by preliminary fractionation of the lipids, and analysis of their fatty acids by gas chromatography. Part of this work has been briefly reported (Macfarlane, Gray & Wheeldon, 1960).

METHODS

Analytical methods. Methods used for P, N, amino N, inositol, fatty acid ester (colorimetric), iodine values, cholesterol, phosphomonoester and ester phosphatide were those used by Gray & Macfarlane (1958). Paper chromatography of water-soluble phosphoric esters obtained by mild alkaline hydrolysis (Dawson, 1954) was done on acid-washed Whatman no. 1 papers by the ascending method, with phenol saturated with 1% ammonia in water, or *tert.*-butyl alcohol–water 62:38 (v/v), trichloroacetic acid 10% (w/v). The P content of spots was estimated according to Bartlett (1959). The spots were identified with authentic markers. Chromatography on silicic acid paper was done according to Marinetti, Erbland & Kochen (1957).

Chromatography of lipid extracts on silicic acid. This was done as in Gray & Macfarlane (1958), by elution with increasing concentrations of methanol in chloroform.

Preparation of methyl esters of fatty acids. Phospholipid fractions (4–5 mg. of P) were saponified in aqueous N-NaOH at room temp. under N_2 for 24 hr. in the dark. Neutral-lipid samples were saponified by refluxing for 3 hr. in 0·5 N-NaOH in 95% ethanol; the hydrolysate was neutralized with HCl, the ethanol removed *in vacuo* and the aqueous mixture extracted with light petroleum (60–80°) before acidification and extraction of fatty acids. The acids were converted into methyl esters by refluxing in 2–3 ml. of methanolic 0·8 N-HCl for 3–4 hr.

Gas chromatography of the methyl esters. This was done as described by Gray (1960). The information necessary to identify the unsaturated acids was obtained by chromatographing the mixed fatty acid methyl esters of ox-liver kephalin and lecithin on Apiezon L and Reoplex 400 (James, 1959). It was therefore possible to identify the rat-liver acids by chromatography on the Reoplex 400 phase only. A chromatogram was also run after bromination as a check on the positions of unsaturated acids.

EXPERIMENTAL AND RESULTS

Preparation of sub-cellular particles. Rats of the Chester Beatty strain (350–400 g.) were used; these were kept on a stock diet (Rank no. 41 b), with small regular supplements of milk, cod-liver oil and Marmite. Three rats at a time were bled, and the liver was homogenized in a Craigie (1949) grinder in 0·25 M-sucrose (10 ml./g. of tissue). The homogenate was centrifuged successively at (*a*) 600 ***g*** for 10 min. to deposit nuclei, red cells and unruptured liver cells; (*b*) 12 000 ***g*** for 10 min., the grey–brown deposit being designated as mitochondria; (*c*) 78 000 (mean) ***g*** for 90 min., the red pellet being designated as microsomes.

Extraction of lipid. Samples of whole liver were minced finely with scissors and extracted with 19 ml. of chloroform–methanol (2:1, v/v)/g. of tissue. The mitochondria and microsomes were suspended in a small volume of water and stirred with 19 vol. of chloroform–methanol (2:1, v/v) at room temp. for a few minutes. The extract after filtration was washed twice with 0·2 vol. of 2 mM-$CaCl_2$: the chloroform layer was taken to dryness on a rotary evaporator at 30° *in vacuo* and the residue was dissolved in chloroform and stored in N_2 at −10°. The final supernatant after centrifuging of the homogenate at 78 000 ***g*** was evaporated to a small volume and extracted similarly. Extracts from a total of 120 g. of liver were pooled for fractionation.

Yields. Some analytical results for the extracts from whole liver, mitochondria and microsomes are given in Table 1. Considerable numbers of unruptured cells were present in the first deposit, so the recovery of particles was not quantitative; about 60% of the original lipid P was present in the mitochondria and microsomes recovered: the extract from the supernatant contained only 10 μg. of lipid P/g. of liver extracted, and was not investigated.

Table 1. *Analyses of lipid extracts from rat liver*

Lipid extract from	Whole liver	Mitochondria	Microsomes
Wt. of liver extracted (g.) ...	18	(from) 120	(from) 120
Lipid extract			
Dry wt. (g.)	1·04	1·62	1·13
P (mg.)	21	49	35
Amino N (mg.)	3·3	8·0	4·4
Total cholesterol (mg.)	21	31	21
P (% on dry wt.)	2·0	3·0	3·1

Table 2. *Phospholipid fractions from mitochondrial lipid*

Abbreviations: GPGPG, polyglycerophosphate from cardiolipin; GPS, glycerophosphorylserine; GPE, glycerophosphorylethanolamine; GPI, glycerophosphorylinositol; GPC, glycerophosphorylcholine.

Fraction	MA	MB	MC	MD	ME	MF
Eluate nos.	7–13	14–18	19–31	32–37	38–45	46–60
Eluent: $CHCl_3$–methanol (v/v)	19:1	4:1	4:1	3:2	3:2	1:4
Wt. (mg.)	123	408	83	167	191	161
P (mg.)	3·8	15·2	2·8	6·1	7·1	6·2
P (% on dry wt.)	3·1	3·7	3·4	3·7	3·7	3·8
Molar ratio (P = 100)						
Amino N	6	75	20	5	Nil	Nil
Fatty acid ester	197	190	177	195	193	188
Inositol	—	12	22	Nil	—	—
Double bonds/mol. of fatty acid	2·0	2·2	—	2·0	1·7	1·6
P in fraction (% of total)	9·2	37·0	6·8	14·8	17·2	15·0
Phosphoric esters*	GPGPG	GPS	GPS	GPC	GPC	GPC
	GPS	GPE	GPE	—	—	—
	—	GPI	GPI	—	—	—
	—	—	GPC	—	—	—

* By paper chromatography of water-soluble P esters formed by mild alkaline hydrolysis.

Fractionation of lipid extracts on silicic acid

Mitochondrial lipid. The material (46 mg. of P) in chloroform was placed on a column of 40 g. of silicic acid. Passage of chloroform eluted 230 mg. of lipid free from P (neutral-lipid fraction), containing 600 μequiv. of fatty acid ester and 2 mg. of cholesterol. Elution was continued with chloroform–methanol mixtures, 20 ml. fractions being collected, which were pooled on the basis of the P and amino-N content and further analysed (Table 2). The first fractions eluted after changing to chloroform–methanol (19:1, v/v) contained 46 mg. of lipid free from P, with about 15 μmoles of fatty acid ester and 19 mg. of cholesterol. This was followed by a phospholipid band low in amino N with 4 mg. of cholesterol (fraction MA); on silicic acid paper this gave a large cardiolipin spot and weak phosphatidylethanolamine and phosphatidylserine spots; the deacylated ester also gave a spot identical with that from cardiolipin. The main kephalin band (fraction MB) containing phosphatidylserine and phosphatidylethanolamine was eluted rapidly in chloroform–methanol (4:1, v/v), with the inositide overlapping. Owing presumably to the high degree of unsaturation the lecithin band, rather unusually, was eluted with this solvent, the front just overlapping into the tail (fraction MC) of the kephalin and inositide. The lecithin band was broad and successive eluates were pooled into three fractions MD, ME and MF with decreasing iodine values: the P in these fractions was all present as fatty acid ester phosphatide and was hydrolysed by *Clostridium welchii* lecithinase, and on paper chromatography the deacylated ester gave only a glycerylphosphorylcholine spot. About 97 % of the P was recovered from the column after passage of chloroform–methanol (1:4); sphingomyelin was not detected, but its presence in the original extract was not excluded. The kephalin and lecithin fractions were virtually free from plasmalogens.

The distribution of P in the various phospholipids of the mitochondria was computed from the analyses in Table 2 as follows: cardiolipin 9; phosphatidylserine and phosphatidylethanolamine 31; phosphatidylinositol 6; lecithin 51 % of the total P.

Microsomal lipid. The material (34 mg. of P) was chromatographed in a similar way on 28 g. of silicic acid. The chloroform eluate (neutral-lipid fraction) contained 72 mg. of lipid with about 160 μequiv. of fatty acid ester and 1 mg. of cholesterol. With chloroform–methanol (19:1, v/v) a small band (0·36 mg. of P) with an amino N:P ratio of 0·06:1·0 was first eluted. This band contained 71 mg. of material, including about 20 mg. of total cholesterol; the deacylated product gave on paper-

chromatography spots corresponding to glycerophosphate and the polyglycerophosphate derived from cardiolipin. This band was immediately followed by the front of the kephalin band eluted with the same solvent. The remainder of the fractionation was very similar to that of the mitochondrial extract. Plasmalogens were virtually absent. The analyses of the fractions are given in Table 3. The distribution of P in the microsomal phospholipid was computed as follows: cardiolipin and phosphatidic acids, not more than 1; phosphatidylserine and phosphatidylethanolamine, 26; phosphatidylinositol, 10; lecithin 62% of the total P.

Fatty acid composition of fractions

Table 4 shows the fatty acid composition of: (*a*) the neutral lipid, mainly glycerides, in the chloroform eluates; (*b*) the main kephalin fraction; (*c*) the lecithin, as the weighted mean of the three fractions analysed; (*d*) the separate fractions of the mitochondrial lecithin. Fig. 1 shows the gas chromatograms of the fatty acids of the three lecithin fractions from the microsomes.

There is a striking similarity between the corresponding fractions of the mitochondria and microsomes. The neutral lipid contained more than 50%

Table 3. *Phospholipid fractions from microsomal lipids*

For explanation of abbreviations see Table 2.

Fraction	XA	XB	XC	XD	XE	XF	XG
Eluate nos.	1–5	6–11	12–14	15–26	27–30	31–36	37–55
Eluent: $CHCl_3$–methanol (v/v)	19:1	19:1	4:1	4:1	3:2	3:2	3:2, 1:4
Wt. (mg.)	71	65	211	75	192	179	192
P (mg.)	0·36	2·1	8·1	2·7	7·0	6·6	7·5
P (% on dry wt.)	0·5	3·2	3·8	3·6	3·6	3·7	3·9
Molar ratio: (P = 100)							
Amino N	6	77	76	30	5	Nil	Nil
Fatty acid ester	—	200	190	208	190	182	190
Inositol	—	24	22	34	—	—	—
Double bonds/mol. of fatty acid	—	2·1	2·0	0·8	2·2	1·5	1·0
P in fraction (% of total)	1·1	6·2	23·8	7·8	20·6	19·4	21·9
Phosphoric esters*	GP	GPE	—	GPE	—	—	—
	GPGPG	GPS	GPE	GPI	GPC	GPC	GPC
	—	GPI	GPI	GPC	—	—	—

* By paper chromatography of water-soluble P esters formed by mild alkaline hydrolysis.

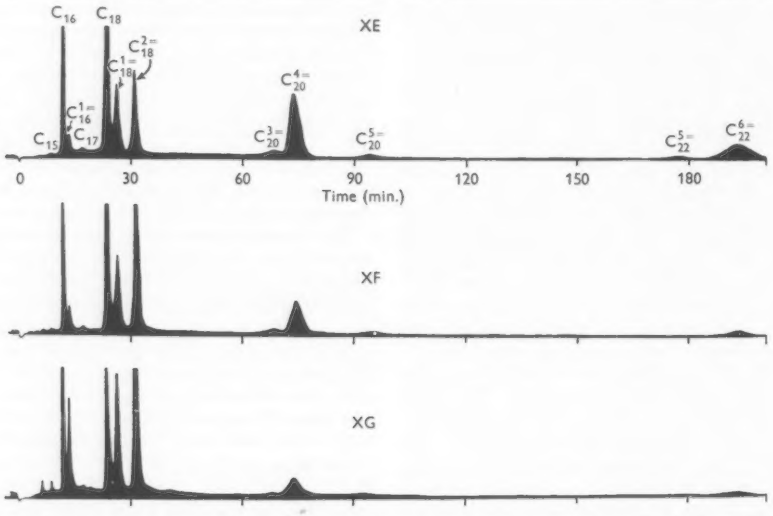

Fig. 1. Gas chromatograms of methyl esters of fatty acids from successive lecithin fractions XE, XF, XG of rat-liver microsomes (cf. Table 3).

Table 4. *Fatty acid composition of neutral-lipid, kephalin and lecithin fractions from rat-liver microsomes and mitochondria*

Values are expressed as percentages of total fatty acid methyl esters. Chromatography on Reoplex 400 at 190°.

		Neutral lipid		Main kephalin fraction		Lecithin*		Lecithin fractions		
								Mitochondria		
Fatty acid	Designation	Mitochondria $CHCl_3$ eluate	Microsomes $CHCl_3$ eluate	Mitochondria MC	Microsomes XB	Mitochondria MD–F	Microsomes XE–G	MD	ME	MF
Undecanoic	11:0	0·3	—	—	—	—	—	—	—	—
Lauric	12:0	—	—	0·4	—	—	—	—	—	—
Myristic	14:0	0·8	0·9	—	—	0·3	0·2	0·2	0·1	0·6
Pentadecanoic	15:0	0·2	0·5	0·2	—	0·2	0·2	0·2	0·1	0·3
Palmitic	16:0	23·9	22·5	17·5	17·5	13·2	13·2	11·0	13·3	15·2
Palmitoleic	16:1	10·1	11·6	1·0	0·7	3·5	3·7	1·4	3·2	6·2
Heptadecanoic	17:0	0·7	1·0	0·6	0·3	0·6	0·9	0·4	1·0	0·5
—	17:?	0·9	0·9	0·3	—	0·1	0·2	—	0·3	—
Stearic	18:0	2·1	2·4	30·4	34·5	21·9	24·9	22·2	21·6	21·9
Oleic	18:1	44·9	40·5	4·8	4·4	14·1	12·1	11·5	13·6	17·2
Linoleic	18:2	14·6	16·3	4·3	4·2	19·6	19·5	13·7	20·8	24·2
Linolenic	18:3	—	0·5	—	—	—	—	—	—	—
Nonadecanoic	19:0	—	0·8	—	—	0·5	—	1·1	—	0·5
Eicosatrienoic	20:3	—	—	0·8	1·3	1·5	1·2	2·0	1·4	1·3
Arachidonic	20:4	0·9	1·8	21·0	23·0	13·8	12·6	18·1	14·3	8·8
Eicosapentaenoic	20:5	0·5	—	2·4	0·6	1·9	1·4	1·1	3·6	0·9
Docosapentaenoic	22:5	—	—	2·0	2·0	1·2	0·4	2·9	0·9	—
Docosahexaenoic	22:6	—	—	14·0	11·7	7·9	7·7	14·3	7·3	2·2
Saturated acids	—	28·9	29·0	50·2	53·6	38·3	40·8	35·1	36·4	38·6
Monoenoic acids	—	55·0	52·1	5·8	5·1	14·7	15·8	12·9	16·8	23·4
C_{20}–C_{22} acids	—	1·4	1·8	40·2	38·6	26·3	23·3	38·4	27·5	13·2

* Weighted mean.

of monoenoic acids, very small amounts of C_{20}–C_{22} polyenoic acids and palmitic acid as the predominant saturated acid. The analyses of the kephalin bands, which contained 10–20% of phosphatidylinositol as well as phosphatidylserine and phosphatidylethanolamine, give a general picture of the components as compounds rich in C_{20}–C_{22} polyenoic acids (40%) and low in oleic acid and linoleic acid. This kephalin differs from that of ox-heart kephalin in three main respects: (1) the content of palmitic acid (20% against 4%); (2) the high content of arachidonic acid (21% against 12%); (3) the remarkable content (12–14%) of docosahexaenoic acid; C_{22} acids were not detected in ox-heart kephalin (Gray, 1960).

The lecithin fraction of the liver particles is even more remarkable in its high content (25%) of C_{20}–C_{22} polyenoic acids, including 8% of docosahexaenoic acid, for these acids are present in very small amounts, if at all, in heart-muscle lecithin and egg lecithin (Gray, 1960; Hawke, 1959); also, unusually, there is more stearic acid than palmitic acid. The successive lecithin fractions MD, ME and MF all had about the same proportion of saturated acids; the decrease in iodine values throughout the band is due to the substitution of oleic acid and linoleic acid for arachidonic acid and docosahexaenoic acid.

Linolenic acid was present (0·5%) in the microsomal neutral lipid, but was not detected in any of the phospholipid fractions. C_{15} and C_{17} acids were present in small amounts in all the fractions and C_{20} trienoic and pentaenoic acids and C_{22} pentaenoic acids in the kephalin and lecithin. In contrast with ox liver, C_{22} hexaenoic acid preponderates over the C_{22} pentaenoic acid in rat liver.

DISCUSSION

The only striking difference between the phospholipids of the mitochondria of rat liver and those of the microsomes is in the content of cardiolipin, which is about 9% of the total in mitochondria and not more than 1% in the microsomes. Marinetti, Erbland & Stotz (1958) found that mitochondria of pig-heart ventricle contained significantly more polyglycerophosphatide than did the microsomes. Getz & Bartley (1959) isolated the polyglycerophosphatide from rat-liver mitochondria and found the fatty acid composition was similar to that of cardiolipin from heart muscle.

The neutral-lipid fractions from mitochondria and microsomes are very similar in fatty acid composition, and so are the corresponding phospholipid fractions. The results are broadly in agreement with those of Clément *et al.* (1955, 1956) but the content of hexaenoic acids in the phospholipids is considerably higher.

The very high content of 'essential' fatty acids, reckoned as the sum of linoleic acid and C_{20}–C_{22} polyenoic acids, and particularly of the polyenoic acids, in the phospholipids is of interest in several respects. It is not clear what part phospholipids play in the metabolic activity of the subcellular particles, but it is known from the action of lecithinases that the integrity of the lecithin in mitochondria is necessary for the succinoxidase activity (Macfarlane, 1950; Nygaard & Sumner, 1953; Macfarlane & Datta, 1954) and doubtless the other phospholipids are also of functional significance. A dietary deficiency of essential fatty acids would presumably limit the rate of production of mitochondria and microsomes and hence the rate of growth, though it would not necessarily affect the metabolic activity of the particles already present.

Another interesting aspect is the susceptibility of the highly unsaturated acids to peroxidation. Tappel & Zalkin (1959), for example, have shown that lipid peroxidation in isolated mitochondria is accompanied by deterioration in enzymic activity. Horgan, Philpot, Porter & Roodyn (1957) investigated the toxicity of auto-oxidized linoleic acid, and the formation of peroxides *in vivo* in relation to the toxicity of radiation, and suggested that chain auto-oxidation of essential fatty acids would produce lethal doses of peroxide in sites not protected by vitamin E. The toxicity may possibly be referred to the direct inhibition by peroxidation of the metabolic activity of the mitochondria or microsomes.

SUMMARY

1. The lipid extracted from the mitochondria of rat liver was similar to that from the microsomes except that the mitochondria contained significantly more cardiolipin than the microsomes.

2. The fatty acid composition of the neutral-lipid, kephalin and lecithin fractions of the mitochondria and microsomes of rat liver was determined by gas chromatography. The corresponding fractions from the two kinds of particles were very similar.

3. Monoenoic acids (53%), palmitic acid (23%) and linoleic acid (15%) were the main acids in the neutral lipid.

4. The lecithin and kephalin fractions contained respectively 25% and 40% of the fatty acids as C_{20} and C_{22} acids, with a high proportion of arachidonic acid and docosahexaenoic acid.

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REFERENCES

Bartlett, G. R. (1959). *J. biol. Chem.* **234**, 466.

Clément, G., Clément, J. & Le Breton, E. (1956). In *Biochemical Problems of Lipids*, p. 385. Ed. by Popják, G. & Le Breton, E. London: Butterworths Scientific Publications.

Clément, G., Haimovici, D. & Le Breton, E. (1955). *C.R. Acad. Sci., Paris*, **240**, 1827.
Craigie, J. (1949). *Brit. J. Cancer*, **3**, 439.
Dawson, R. M. C. (1954). *Biochim. biophys. Acta*, **14**, 374.
Getz, G. S. & Bartley, W. (1959). *Nature, Lond.*, **184**, 1229.
Gray, G. M. (1960). *Biochem. J.* **77**, 82.
Gray, G. M. & Macfarlane, M. G. (1958). *Biochem. J.* **70**, 409.
Hawke, J. C. (1959). *Biochem. J.* **71**, 588.
Horgan, V. J., Philpot, J. St L., Porter, B. W. & Roodyn, D. B. (1957). *Biochem. J.* **67**, 551.
James, A. T. (1959). *J. Chromat.* **2**, 552.
Macfarlane, M. G. (1950). *Biochem. J.* **47**, 29P.
Macfarlane, M. G. & Datta, N. (1954). *Brit. J. exp. Path.* **35**, 191.
Macfarlane, M. G., Gray, G. M. & Wheeldon, L. W. (1960). *Biochem. J.* **74**, 43P.
Marinetti, G. V., Erbland, J. & Kochen, J. (1957). *Fed. Proc.* **16**, 837.
Marinetti, G. V., Erbland, J. & Stotz, E. (1958). *J. biol. Chem.* **233**, 562.
Nygaard, A. P. & Sumner, J. B. (1953). *J. biol. Chem.* **200**, 723.
Tappel, A. L. & Zalkin, H. (1959). *Arch. Biochem. Biophys.* **80**, 326.

Biochem. J. (1960) **77**, 631

Improved Fractionations of Arginine-Rich Histones from Calf Thymus

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It has been shown by a variety of methods that calf-thymus histones can be separated into fractions relatively rich in arginine (Grégoire & Limozin, 1954; Daly & Mirsky, 1955; Davison & Shooter, 1956) and in lysine (Stedman & Stedman, 1951; Davison & Butler, 1954; Crampton, Moore & Stein, 1955; Daly & Mirsky, 1955). Further chromatographic separation on Amberlite IRC-50 resin columns was also achieved by Luck, Rasmussen, Satake & Tsvetikov (1958) with a gradient of guanidinium chloride for elution. Davison (1957) first reported the use of carboxymethylcellulose columns with a salt gradient at pH 4·2 which gave two main peaks, in addition to an initial small fraction very rich in aspartic and glutamic acids. The first main peak was a lysine-rich histone and the second, larger, peak was heterogeneous, with a higher arginine content in the part eluting more slowly. Later it was possible to elute histone as two fractions from carboxymethylcellulose columns with dilute hydrochloric acid, the fractionation being followed by *N*-terminal amino acid analysis (Phillips & Johns, 1959). The two fractions had mainly proline and mainly alanine *N*-terminal groups respectively. These two systems of elution have now been combined to give an improved fractionation into three parts. A bulk fractionation process has also been worked out, which readily gives arginine-rich histones, with alanine as virtually the sole *N*-terminal group. This method is based on the extraction of the whole tissue with acid in the presence of a high concentration of ethanol. It was thought that under these conditions the action of tissue proteinases, such as that shown to be present in calf thymus (Phillips & Johns, 1959), would be prevented. It was realized that proteins other than histones would be dissolved from the tissue in this procedure, but judging by the analytical results on the product it is thought that these have been eliminated. This paper is mainly confined to the study of the arginine-rich histones obtained by the chromatographic and the extraction procedures.

EXPERIMENTAL AND RESULTS

Improved chromatographic separation on carboxymethylcellulose. The histone was prepared from calf-thymus glands by the methods previously described, with slightly acidified sodium chloride washes (Phillips & Johns, 1959). The histone (100 mg.) was dissolved in 20 ml. of buffer, pH 4·2, containing 0·1 M-acetic acid and 0·03 M-sodium hydroxide, and applied to carboxymethylcellulose in the form of a column, 14 cm. long and 2 cm. in diameter, which had been prepared by allowing a slurry of 5 g. of carboxymethylcellulose in the same buffer to settle in the chromatographic tube under gravity. Fractions of 3–5 ml. were collected automatically at a flow rate of about 0·5 ml./min. and the progress of the elution was followed by measuring the extinction of the fractions at 278 mμ. Elution was carried out in four steps as follows: (1) a buffer, pH 4·2, containing 0·1 M-acetic acid and 0·03 M-sodium hydroxide; (2) a buffer, pH 4·2, containing 0·17 M-acetic acid, 0·051 M-sodium hydroxide

and 0·42 M-sodium chloride; (3) 0·01 N-HCl and (4) 0·02 N-HCl.

The result of a fractionation with whole histone preparation H 42 is shown in Fig. 1. The peak fX contained only about 0·5 % of the total nitrogen taken and had an amino acid composition very like that of the initial contaminant peak reported by Davison (1957). Step (2) then removed the very lysine-rich histones as two small peaks f1A and f1B. Histones f2 and f3 were then eluted in steps (3) and (4). Table 1 gives some of the total amino acid and *N*-terminal group analyses of fractions f1A, f2 and f3 as determined by methods previously described (Phillips, 1958; Phillips & Johns, 1959). The lysine-rich histone f1B had a lower lysine to arginine ratio than histone f1A but was otherwise very similar in composition. The yield of the arginine-rich histone, f3, was about 20 % of the whole histone taken for chromatography.

When, instead of applying whole histone to the carboxymethylcellulose column, either the 0·01 N-HCl or the 0·02 N-HCl fractions were used [prepared

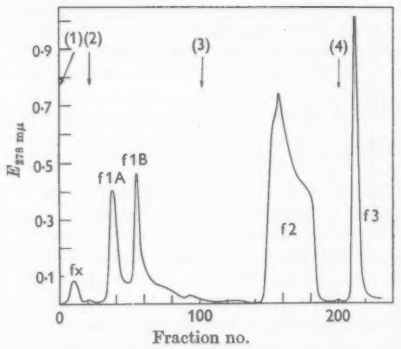

Fig. 1. Effluent diagram of calf-thymus histones (510 mg.) chromatographed on a carboxymethylcellulose column (20 cm. × 2·4 cm.). The histone was applied to the column in 20 ml. of 0·1 M-sodium acetate buffer, pH 4·2, and eluted with: (1) the same buffer; (2) sodium acetate–sodium chloride buffer, pH 4·2 [for (1) and (2) see text]; (3) 0·01 N-HCl and (4) 0·02 N-HCl. The average fraction size was 4 ml.

Table 1. *Composition of histones from chromatography and acid–ethanol extraction*

The amino acids are expressed as moles/100 moles of all amino acids found and no correction has been made for the hydrolytic losses of amino acids. Amide was not determined. The analytical values for the starting material are the averages of results from histone preparations HV5 and H 42, used for preparing the column fractions f1A, f2 and f3. The 70 and 80 % ethanol preparations are the same as preparations 70(1) and 80(3) respectively in Table 2. The proportions of *N*-terminal groups are molar percentages of all such groups found.

Histone fraction (for origin, see text)	Starting material	Column fractions: f1A (average of 3)	Column fractions: f2 (average of 3)	Column fractions: f3 (average of 2)	70 % Ethanol preparation	80 % Ethanol preparation	70 % Ethanol preparation. Column fraction f3
Amino acid							
Aspartic acid } A	14·2	8·0	14·3	16·9	5·7	4·2	4·3
Glutamic acid } A					10·5	12·0	12·0
Glycine	8·5	7·2	10·3	6·6	7·5	6·1	5·8
Alanine	13·8	23·6	11·1	13·0	13·1	13·7	13·9
Valine	6·4	4·8	7·5	5·2	5·6	5·7	5·3
Leucine + isoleucine	10·6	5·4	12·7	13·8	13·7	13·6	13·3
Phenylalanine	1·9	0·7	1·5	2·9	2·2	2·4	3·0
Tyrosine	1·7	0·5	2·7	0·5	1·8	1·3	0·8
Serine	5·2	6·1	5·2	4·4	4·0	4·2	4·6
Threonine	5·8	5·6	5·4	6·2	6·1	6·9	6·6
Proline	5·1	8·9	3·6	4·9	5·0	4·7	4·9
Histidine }	2·0	0·4	2·7	2·2	2·2	2·2	1·8
Lysine } B	15·6	25·9	13·1	10·3	9·9	9·0	9·0
Arginine } B	9·2	2·9	9·8	13·2	12·6	14·0	14·7
Lysine:arginine	1·70	9·0	1·34	0·78	0·78	0·64	0·61
Basic:acidic; B/A	1·89	3·6	1·79	1·52	1·52	1·54	1·56
N-terminal groups							
Alanine	34*	—	10	69	85	96	90
Proline	51*	—	70	14	6	3	4
Others	15*	—	20	17	9	1	6
Wt. (g./mole of *N*-terminal groups)	27 000	—	28 500	17 800	22 300	18 000	17 600

* Sample HV5 only.

from a column run with acid only, see Phillips & Johns (1959)], then the acetate–chloride step (2) removed a small peak of lysine-rich material in both cases. The main fraction was then eluted by step (3) or step (4) respectively.

Preparation of arginine-rich histones by extraction with acid–ethanol. Calf thymus (100 g.) was homogenized with 700 ml. of ethanol–water (70:30, v/v) for 2 min. in a Waring Blendor (the concentration of ethanol used in this step was always the same as that used in the subsequent extraction). The homogenate was centrifuged at 1000 *g* for 20 min. and the supernatant was discarded. The sediment was washed once more by this method and then extracted in a ball mill with 200 ml. of ethanol–N-HCl–water (70:25:5, by vol.). This extraction was carried out at 4° for 20 hr., the suspension was centrifuged and the sediment again extracted with 100 ml. of the solvent for 4 hr. followed by a final extraction with 100 ml. for 2 hr. The combined supernatants were dialysed three times for 4 hr. at 4° against 600 ml. of ethanol. The precipitate which formed accounted for 33% of the nitrogen extracted, while 58% remained in the alcoholic supernatant and only 9% diffused out of the bag. After centrifuging, the precipitate was washed with ethanol, then with acetone and dried under vacuum. The vacuum was released every few minutes and any lumps of material were broken up with a glass rod. By drying in this manner, a fine white powder was obtained which was readily soluble in water. The average yield was 0·58 g. or 24% of the usual yield of all histones from calf thymus.

The protein remaining in the supernatant in the dialysis bag had the composition of a histone, containing 24 moles of basic amino acids/100 moles of all amino acids, with a lysine/arginine ratio of 1:1.

The effect of varying the concentration of ethanol used for the extraction from 10 to 98% (v/v) has been studied (in all cases the solvents were 0·25N with respect to HCl). The results of these experiments and the preparation described above are shown in Table 2, in which the yield, the *N*-terminal groups, the lysine:arginine ratio and the molar proportion of all basic amino acids are also given. In one preparation with ethanol–1·25N-HCl (80:20, v/v), the final precipitate was redissolved in fresh

Table 2. *Yield and properties of histones prepared by acid–ethanol extractions*

N-Terminal groups are expressed as molar percentages of all such groups found. Those other than alanine and proline were glycine, valine, leucine (and isoleucine), serine, threonine, aspartic acid and glutamic acid. The basic acids referred to are arginine, histidine and lysine.

Ethanol concentration (%, by vol.). (Solutions all 0·25N with respect to HCl)	Yield of histone (mg./10 g. of calf thymus)	*N*-Terminal groups			Basic acids (% of moles of all amino acids)	Moles of lysine / Moles of arginine
		Proline	Alanine	Others		
98	0	—	—	—	—	—
90	0	—	—	—	—	—
80(1)	48	5	86	9	25·5	0·76
80(2)	42	7	86	7	—	—
70(1)	66	6	85	9	24·7	0·78
70(2)	50	8	83	9	—	—
60	86	9	78	13	26·7	1·1
50	115	13	76	11	22·9	1·7
40	121	37	44	19	23·1	1·6
30	118	36	46	18	24·8	1·9
20	149	43	44	13	24·6	1·8
10	120	39	50	11	24·5	1·9
0 (i.e. 0·25N-HCl)	240	41	32	27	26·3	1·9
80(3) Redissolved and redialysed	33	3	96	1	25·2	0·64

Table 3. *Composition of histones obtained by successive acid–ethanol extractions*

N-Terminal groups are expressed as molar percentages of all the *N*-terminal groups found. The molecular weight is the g. wt. of histone/mole of *N*-terminal groups. The values for total lysine and arginine are molar percentages of all the amino acids found in these histones.

Ethanol concentration (%, by vol.). (Solutions all 0·25N with respect to HCl)	Yield (mg./100 g. of calf thymus)	*N*-Terminal groups		Molecular weight	Total amino acids	
		Proline	Alanine		Lysine	Arginine
80	480	5	86	17 800	9	14
Subsequent extraction with 50	60	18	67	22 000	12	10·5
Subsequent extraction with 10	320	40	31	46 000	20	5

Table 4. *Further properties of arginine-rich histones*

The molar ratios lysine–arginine are calculated from the analyses of complete acid hydrolysates of the fractions. The *C*-terminal amino acid values are expressed as moles/18 000 weight of histone (the weight containing 1 mole of *N*-terminal groups).

Histone fraction (for origin, see text and Table 2) ...		70(1)		80(3)	
Elution step		Amount eluted (%, by wt.)	Moles of lysine / Moles of arginine	Amount eluted (%, by wt.)	Moles of lysine / Moles of arginine
Carboxymethylcellulose column chromatography	(1)	Trace	—	0	—
	(2)	10	1·02	0	—
	(3)	42	0·93	16	0·87
	(4)	48	0·61	84	0·64
C-terminal groups		Moles/18 000 g. of histone		Moles/18 000 g. of histone	
By hydrazinolysis	Alanine	0·2		0·6	
	Others	0·1		0·1	
By carboxypeptidase	Alanine	0·4	22 hr. digestion	0·3	28 hr. digestion
	Others	0·1		0·05	

solvent and dialysed against ethanol. The yield fell but the protein had 96% of alanine *N*-terminal groups [cf. 80(1) and 80(2) with 80(3) in Table 2].

In another experiment the extraction of one sediment with successively lower concentrations of ethanol (all 0·25N with respect to HCl) extracted histones of quite different composition, the final one, with 10% ethanol, being a lysine-rich histone containing 20 moles of lysine/100 moles of all amino acids. The results of this procedure are shown in Table 3.

Other properties of the arginine-rich histones. The materials which have been most studied were prepared by 70 and 80% ethanol extractions [samples 70(1) and 80(3) of Table 2]. These have been examined as follows:

(*a*) Starch-gel electrophoresis. The method of Smithies (1955) was used, but the gel was prepared in 0·01N-HCl to avoid aggregation of the histone and to suppress alkaline-proteinase activity. The sample was dissolved in 0·01N-HCl and applied to the gel by the filter-paper technique. Both samples of arginine-rich histone gave two major and two minor bands staining with naphthalene black. Whole calf-thymus histone under these conditions gave ten bands, three of them very faint. Both the filter-paper technique and the staining method are as described by Smithies (1955).

(*b*) Alkaline-proteinase activity. The method of Phillips & Johns (1959) was used. The *N*-acetyl-L-tyrosine ethyl ester was digested in pH 7·7 buffer, since at this pH the autolysis of the substrate in 16 hr. at 40° was negligible. Sample 70(1) showed activity, but none was detected in sample 80(3).

(*c*) Chromatography on carboxymethylcellulose. The system with four eluents described above was used. The results are given in Table 4.

(*d*) *C*-terminal amino acids. The action of carboxypeptidase (Armour and Co. Ltd.) was studied by the method of Fraenkel-Conrat, Harris & Levy (1955). Both the enzyme and the histone were separately treated for 1·5 hr. at 20° and pH 7·7 with mM (final concentration) di*iso*propyl phosphorofluoridate, to inactivate proteinases. The solutions were then mixed and carboxypeptidase action was allowed to proceed at 40°. The *N*-terminal alanine residue remained unchanged during the digestion. The hydrazinolysis method used was mainly that of Niu & Fraenkel-Conrat (1955), but 95% hydrazine was used and the reaction time kept at 10 hr. at 100° throughout. The results of the *C*-terminal amino acid experiments are also given in Table 4.

DISCUSSION

The combination of the acetate–sodium chloride and acid elutions from carboxymethylcellulose columns clearly separates calf-thymus histones into (*a*) very lysine-rich histone with a lysine:arginine molar ratio of 6–9:1, (*b*) slightly lysine-rich histone with a lysine:arginine ratio of about 1·3:1 and having mainly proline *N*-terminal groups, and (*c*) arginine-rich histone, with a lysine:arginine ratio of about 0·8:1 and bearing mainly alanine *N*-terminal groups. The analysis of the lysine-rich histone (see f1A in Table 1) approximates in composition to the lysine-rich histone of other authors (Davison & Butler, 1954, and Crampton *et al.* 1955). The analysis of group (*b*), the slightly lysine-rich histone, agrees fairly well with that of the histone II reported by Luck *et al.* (1958). The groups (*b*) and (*c*) are richer in arginine than the corresponding fractions reported by Phillips & Johns (1959), owing to the removal here of lysine-rich histone from both by the

sodium acetate–sodium chloride elution step. The amino acid analyses of group (c) are also similar to those reported by Mauritzen & Stedman (1959) for the β-histones of three fowl tissues. The arginine-rich group of histones, which chiefly concerns us here, can however be more conveniently prepared by the acid–ethanol extraction method described above. The acid–ethanol extraction of thymus previously washed with aqueous ethanol undoubtedly dissolved some non-histone material from the tissue, particularly with the lower ethanol concentrations. However, the subsequent dialysis (against ethanol) precipitated only basic proteins, as is shown by the presence of 23–27 moles of basic amino acids in every 100 moles of all acids in these preparations (see Table 2). Whole calf-thymus histone and the carboxymethylcellulose chromatographic fractions obtained from it have 25–29 moles of histidine, lysine and arginine per 100 moles of all amino acids in them (see Table 1).

The arginine-rich histones obtained by the two different methods give very similar amino acid analyses and weights per mole of *N*-terminal residues (see Table 1), and both are obtained in similar yields from calf-thymus tissue. However, the extraction method is better than the column method for this group since the *N*-terminal residue of the extraction product is almost entirely one amino acid, alanine, and the lysine:arginine ratio is lower. This result may be due to the absence of degradation by tissue proteinases in the acid–ethanol preparation. The column method demonstrates some heterogeneity in the materials obtained by the extraction method. This is shown by the presence of a little material eluted by 0·01N-HCl [elution step (3)] having a higher lysine:arginine ratio than the main fraction f3 (see Tables 1 and 4). The heterogeneity of the arginine-rich material is also suggested by the results of *C*-terminal amino acid determinations and the multiple bands observed in starch-gel electrophoresis. Since the *N*-terminal group is unaltered during the carboxypeptidase digestion, we are sure that the alanine released is *C*-terminal. The low yield of *C*-terminal alanine may be due to the presence of other *C*-terminal groups which cannot be split off by carboxypeptidase A or which give very low recoveries in the hydrazine method.

Owing to the small scale of the experiments done so far with starch-gel electrophoresis, the detailed properties of the two major and two minor bands (which are seen even in the best preparation of arginine-rich histone) have not yet been examined. The histones in these bands must presumably all have *N*-terminal alanine residues and they are unlikely, at the acid pH of the electrophoresis, to be artifacts due to aggregation.

SUMMARY

1. Calf-thymus histones, chromatographed on carboxymethylcellulose, gave two very lysine-rich fractions with sodium acetate–sodium chloride elution, a slightly lysine-rich histone with 0·01N-hydrochloric acid elution and finally an arginine-rich histone (f3), eluted with 0·02N-hydrochloric acid.

2. Material with very similar amino acid analysis and chromatographic properties to fraction f3 above could be readily prepared in bulk by extraction of the tissue with ethanol–hydrochloric acid and subsequent dialysis of the extract against ethanol.

3. The arginine-rich histone, which was precipitated, had 1 mole of *N*-terminal groups/18 000 weight, which in reprecipitated material was almost entirely alanine. The chief *C*-terminal group detected by carboxypeptidase and on hydrazinolysis was also alanine, but the yield was only about one-half that of the *N*-terminal group.

4. When lower concentrations of ethanol were used in the extraction the *N*-terminal groups of the final precipitates became more complex. Extraction of washed tissue with successively lower concentrations of ethanol showed that the arginine-rich histones were extracted at higher, and the lysine-rich histones at lower ethanol concentrations. In all cases the solvent used was 0·25N with respect to hydrochloric acid.

5. Starch-gel electrophoresis, column chromatography and perhaps the *C*-terminal group analyses indicated that the arginine-rich material with virtually a single *N*-terminal group still consisted of several components.

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REFERENCES

Crampton, C. F., Moore, S. & Stein, W. H. (1955). *J. biol. Chem.* **215**, 787.

Daly, M. M. & Mirsky, A. E. (1955). *J. gen. Physiol.* **38**, 405.

Davison, P. F. (1957). *Biochem. J.* **66**, 708.

Davison, P. F. & Butler, J. A. V. (1954). *Biochim. biophys. Acta*, **15**, 439.

Davison, P. F. & Shooter, K. V. (1956). *Bull. Soc. chim. Belg.* **65**, 85.

Fraenkel-Conrat, H., Harris, J. I. & Levy, A. L. (1955). *Meth. biochem. Anal.* **2**, 397.
Grégoire, J. & Limozin, M. (1954). *Bull. Soc. Chim. biol., Paris*, **36**, 15.
Luck, J. M., Rasmussen, P. S., Satake, K. & Tsvetikov, A. N. (1958). *J. biol. Chem.* **233**, 1407.
Mauritzen, C. M. & Stedman, E. (1959). *Proc. Roy. Soc.* B, **150**, 299.
Niu, C-I. & Fraenkel-Conrat, H. (1955). *J. Amer. chem. Soc.* **77**, 5882.
Phillips, D. M. P. (1958). *Biochem. J.* **68**, 35.
Phillips, D. M. P. & Johns, E. W. (1959). *Biochem. J.* **72**, 538.
Smithies, O. (1955). *Biochem. J.* **61**, 629.
Stedman, E. & Stedman, E. (1951). *Phil. Trans.* B, **235**, 565.

Biochem. J. (1960) **77**, 636

Polarographic Measurement of Oxygen Uptake by Pea-Root Mitochondria

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Preliminary work on the oxidation of succinate and α-oxoglutarate by pea-root mitochondria showed that the standard manometric method of measuring oxygen uptake was not sufficiently sensitive. A polarographic method was thus developed. It was observed that, whereas the oxidation of α-oxoglutarate as determined manometrically was increased by the addition of diphosphopyridine nucleotide, that measured polarographically was not. This coenzyme also stimulated α-oxoglutarate oxidation (measured polarographically) if the mitochondria were first shaken in a water bath. Similar results with other substrates and cofactors are reported in this paper.

EXPERIMENTAL

Growth of peas and extraction of mitochondria. Peas (*Pisum sativum* L. var. Meteor) were rinsed in ethanol, treated with 10% (w/v) calcium hypochlorite for 30 min. and germinated under sterile conditions in a 5:2 (v/v) vermiculite–water mixture at 26° for 47 hr. Seedlings with roots between 22 and 30 mm. long were selected from those harvested, and a terminal 20 mm. section was excised and placed immediately in ice–water mixture. About 120–140 roots were used in each experiment. Excess of moisture was blotted off the roots, which were weighed and homogenized in a cold glass homogenizer of the Potter–Elvehjem type in 1 vol. of a cold solution containing 0·6M-sucrose and 0·05M-phosphate buffer, pH 6·8. The homogenization was carried out for the minimum length of time needed to ensure disruption of the roots (usually about 10–12 sec.) and in a loose homogenizer (with a clearance of 0·3 mm. round the pestle) which was surrounded by ice.

Unbroken cells, cell walls and nuclei were removed from the homogenate by centrifuging at 1000 *g* for 10 min. at 0°. The supernatant was centrifuged at 15 000 *g* for 15 min. and the precipitate was taken as mitochondria. It was washed by resuspension in 0·5 vol. of the homogenizing medium and centrifuging at 15 000 *g* for 15 min. The mitochondria were finally resuspended in 0·5 vol. of the medium.

In many experiments part of the mitochondrial suspension was shaken for ½ hr. in a test tube in a Warburg bath at 25°, at a speed of 142 oscillations/min. and through a distance of about ½ in.

Measurement of oxygen concentration. Oxygen concentration in the incubation medium was measured polarographically. The circuit of a simple polarograph suitable for such measurements is shown in Fig. 1. The polarizing circuit is shown on the right side. The voltage divider (R_1) enables an e.m.f., derived from the battery (B_1) and measured on the voltmeter (V), to be applied across the electrodes of the cell. The current which passes through the cell is measured on the galvanometer (G). Some current

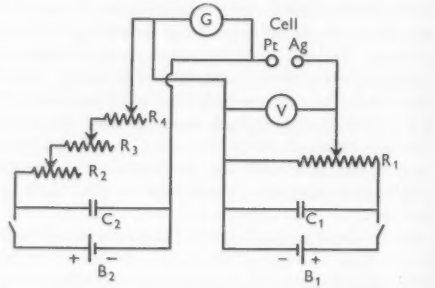

Fig. 1. Circuit of polarograph. B_1, B_2, 1½v dry batteries; C_1, C_2, 50 μF, 50v condensers; G, Scalamp galvanometer 7893/S (W. G. Pye and Co. Ltd., Cambridge); R_1, 125Ω helical potentiometer; R_2, R_3, 5 MΩ linear track potentiometer; R_4, 1 MΩ log track potentiometer; V, 1·5v d.c. voltmeter.

(the residual current) passes through the cell in the absence of oxygen and in order to ensure that the full sensitivity of the galvanometer is being used for measurement of the oxygen-induced current, it is necessary to incorporate the additional circuit shown on the left of Fig. 1. In this, the battery (B_2) applies an e.m.f. across the galvanometer in opposition to that from B_1, and the current, varied by potentiometers R_2, R_3 and R_4, 'backs off' the residual current. Any small fluctuations in the voltage of the batteries is counteracted by the condensers (C_1 and C_2).

The cell used in this work is shown diagrammatically in Fig. 2, and consisted of Pt (negative) and Ag/AgCl (positive) electrodes. Rods of Pt (0·16 mm. diam.) and Ag (0·20 mm. diam.) with Cu wires soldered to them, were immersed in 4 ml. of *n*-butyl methacrylate (L. Light and Co. Ltd.) containing 1% (w/v) of benzoyl peroxide in a polythene specimen tube with an internal diameter of 12·5 mm. The solution was polymerized at 60° overnight and the polythene tube was then stripped away from the solid plastic cylinder, the end of which was turned down in a lathe to expose the maximum surface area of silver. Insulated wires (I) were then soldered to the exposed ends of the copper wires, and with the former uppermost a section of polythene specimen tube (with the base removed) was placed round the cylinder and the cavity filled with *n*-butyl methacrylate (plus catalyst) and polymerized as before. To obtain an Ag/AgCl electrode, the Ag surface was first exposed to conc. HCl for several minutes, then dipped into saturated KCl solution and an e.m.f. of 1·5v was applied for 10 min. with the Ag electrode positive.

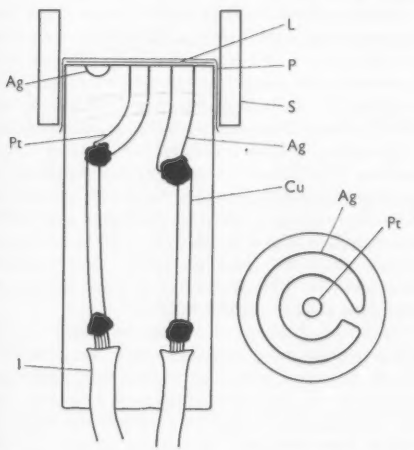

Fig. 2. Section through the complete cell and view of the exposed electrode surface ($\times 1\frac{1}{2}$ approx.). The silver (Ag) and platinum (Pt) electrodes were soldered to copper wires (Cu) and these to insulated wires (I) leading to the polarograph. On the surface of the electrodes were placed a circle of lens tissue (L) and a sheet of polythene (P), held in place by a section of polythene specimen tube (S). Black areas represent solder.

The electrodes were used in a manner similar to those of Bellamy & Bartley (1960). A circle of lens tissue (L; approx. 12·3 mm. diam.) was placed on the electrode surface, moistened with saturated KCl and the whole covered with a disk (22 mm. diam.) of polythene sheet (P) (100 gauge). This polythene sheet was held in place by a section of polythene specimen tube (S), which also formed a vessel for the reactants with a volume of 0·5 ml.

Between 0·5 and 0·8v the oxygen-induced current was approximately constant (0·59 μA). A voltage of 0·7v was selected for further work and the total current at this voltage was 1·04 μA.

For calibration of the electrodes, the piece of tube (S) was replaced by an inverted whole polythene specimen tube containing two small holes for the passage of gas and a larger hole (blocked by a rubber bung) for addition of liquid. Mixtures of air and oxygen-free nitrogen were passed over 0·5 ml. of water in the chamber for 30–45 min. and the steady current obtained after this time was recorded. A straight-line relationship was obtained between current and oxygen concentration in the water (calculated from the gas composition).

Measurement of oxygen uptake. Since the current took some time to reach a steady value, the cell had to be set up and the polarograph switched on $\frac{1}{2}$ hr. before it was required.

All components of the incubation medium (except mitochondria) were placed in the cell, mixed and left for $2\frac{1}{2}$–3 min. to equilibrate and for the current to become constant. The mitochondrial suspension was then added and the mixture was stirred briskly for 20 sec.; a small square of Perspex was placed over the cell and readings were taken on the galvanometer every 5 sec. for 3 min. Addition of the mitochondria and the stirring caused an increase in the current which lasted for 30–40 sec. Care was taken not to touch the polythene membrane, since this gave a much increased current for up to 2 min., probably due to disturbance of the diffusion shell round the Pt electrode. An initial rate of oxygen uptake was obtained graphically from the readings and is expressed as q_{O_2} (μl. of O_2/mg. of N/hr.).

The incubation medium consisted of 0·05M-phosphate buffer, pH 6·8, 0·1 ml. of mitochondrial suspension and substrates (10 mM) and coenzymes as described in the individual experiments, in a total volume of 0·5 ml. All experiments were conducted at room temperature (22–23°).

After each determination the chamber was washed three times with water and mopped out with soft absorbent paper.

Nitrogen estimations. These were done by the Conway-dish method of Sunderland, Heyes & Brown (1957) on the 'acid-insoluble matter' obtained according to the procedure of Werkheiser & Bartley (1957).

Preparation of electron micrographs. The mitochondria were fixed and embedded by the method described by Bellamy (1958), except that for the initial step 0·2 ml. of mitochondrial suspension was added to 1·0 ml. of osmic acid medium and left for 15 min. before centrifuging. Sections were cut and electron micrographs prepared by Mr J. C. W. Crawley.

Special chemicals. The following were used: diphosphopyridine nucleotide (DPN) and thiamine pyrophosphate (L. Light and Co. Ltd., Colnbrook, Bucks.). Cytochrome *c*, adenosine diphosphate (ADP), adenosine triphosphate

(ATP) and coenzyme A (CoA) (Sigma Chemical Co., St Louis, Mo., U.S.A.); bovine-plasma albumin (The Armour Laboratories).

RESULTS

Effect of cytochrome c. In the absence of any added cofactors, the mitochondria oxidized succinate but not α-oxoglutarate (Table 1). On the addition of cytochrome *c* (10 μM) the endogenous rate was the same, but there was a small increase in oxygen uptake in the presence of α-oxoglutarate and the rate with succinate was considerably greater.

Cytochrome *c* could be replaced by bovine-plasma albumin (Table 2). With unshaken mitochondria there was oxidation of succinate in the presence of cytochrome *c*, though this was somewhat less with bovine-plasma albumin. If the mitochondria were shaken in a water bath at 25° for 30 min., the oxygen uptake due to succinate was doubled and was about the same with both cytochrome *c* and bovine-plasma albumin. The protein concentrations in this experiment were 0·2 mg./ml. for cytochrome *c* and 0·5 mg./ml. for bovine-plasma albumin.

Effect of adenosine diphosphate on succinate oxidation. With unshaken mitochondria both the endogenous oxygen uptake and that with succinate were unaffected by the addition of mM-ADP (Table 3). However, ADP stimulated succinate oxidation and, to a lesser extent, the endogenous rate with shaken mitochondria.

Effect of cofactors on α-oxoglutarate oxidation. α-Oxoglutarate was either not oxidized by unshaken mitochondria or, as in Expts. 2, 5 and 6, oxidized only to a small extent (Table 4). However, with shaken mitochondria there was always considerable oxidation of α-oxoglutarate.

DPN (0·1 mM) had no effect on the oxidation of α-oxoglutarate by unshaken mitochondria, but it caused considerable stimulation with shaken mitochondria (Table 4, Expt. 1). A higher DPN concentration (mM) did increase the rate of oxidation of α-oxoglutarate in unshaken mitochondria but decreased that in shaken mitochondria (Table 4, Expt. 2).

Similar effects to that with 0·1 mM-DPN were obtained with ADP (mM), ATP (mM) and thiamine pyrophosphate (0·1 mM) (Table 4, Expts. 3–5). These cofactors caused no increase in α-oxoglutarate oxidation by unshaken mitochondria, but brought about a marked increase with shaken mitochondria.

The effect of CoA was examined at two concentrations. The higher of these (0·1 mM) inhibited the increase in oxygen uptake obtained in the presence of α-oxoglutarate with both unshaken and shaken mitochondria (Table 4, Expt. 6). The lower concentration (0·02 mM) had no effect with unshaken mitochondria, but doubled the total oxygen uptake with shaken mitochondria.

Oxidation of malate and pyruvate. Both L-malate and pyruvate were oxidized either very slowly or not at all when added to unshaken mitochondria (Table 5). As with α-oxoglutarate, however, there was appreciable oxidation of these substrates by shaken mitochondria. If both L-malate and pyruvate were added together, there was an increase in oxygen uptake, small with unshaken mitochondria, but much greater with shaken mitochondria (Table 5, Expt. 1).

DPN had an effect with these substrates similar to that with α-oxoglutarate. It had little or no effect on malate or pyruvate oxidation in unshaken mitochondria, but it brought about a very

Table 1. *Effect of cytochrome* c *on oxygen uptake*

Incubation medium contained 0·05 M-phosphate buffer, pH 6·8, 0·1 ml. of mitochondrial suspension and additions as shown below, in a total volume of 0·5 ml.

Additions	q_{O_2}
None	480
α-Oxoglutarate (10 mM)	480
Succinate (10 mM)	710
Cytochrome *c* (10 μM)	480
Cytochrome *c* (10 μM) + α-oxoglutarate (10 mM)	510
Cytochrome *c* (10 μM) + succinate (10 mM)	810

Table 2. *Replacement of cytochrome* c *by bovine-plasma albumin*

Incubation medium was as given in Table 1. Concn. of bovine-plasma albumin was 0·5 mg./ml.

Additions	q_{O_2} Unshaken mitochondria	q_{O_2} Shaken mitochondria
None	490	400
Succinate (10 mM)	640	720
Cytochrome *c* (10 μM)	480	420
Cytochrome *c* (10 μM) + succinate (10 mM)	1440	2280
Bovine-plasma albumin	400	390
Bovine-plasma albumin + succinate (10 mM)	1330	1940

Table 3. *Effect of adenosine diphosphate on succinate oxidation*

Incubation medium contained 0·05 M-phosphate buffer, pH 6·8, 10 μM-cytochrome *c*, 0·1 ml. of mitochondrial suspension and additions as shown below in a total volume of 0·5 ml.

Additions	q_{O_2} Unshaken mitochondria	q_{O_2} Shaken mitochondria
None	600	600
ADP (mM)	610	790
Succinate (10 mM)	1130	1370
ADP (mM) + succinate (10 mM)	1120	1730

Table 4. *Cofactor requirements for α-oxoglutarate oxidation by unshaken and shaken mitochondria*

Incubation medium was as given in Table 3. α-Oxoglutarate concn. was 10 mM.

Expt. no.	Additions	q_{O_2} Unshaken mitochondria	q_{O_2} Shaken mitochondria
1	None	770	740
	DPN (0·1 mM)	710	610
	α-Oxoglutarate	740	1080
	α-Oxoglutarate + DPN (0·1 mM)	710	1240
2	None	640	600
	DPN (mM)	640	640
	α-Oxoglutarate	670	850
	α-Oxoglutarate + DPN (mM)	750	750
3	None	600	650
	ADP (mM)	580	650
	α-Oxoglutarate	580	720
	α-Oxoglutarate + ADP (mM)	600	920
4	None	560	500
	ATP (mM)	560	500
	α-Oxoglutarate	550	650
	α-Oxoglutarate + ATP (mM)	560	800
5	None	610	680
	TPP (0·1 mM)	650	710
	α-Oxoglutarate	660	910
	α-Oxoglutarate + TPP (0·1 mM)	660	1320
6	None	870	760
	CoA (0·1 mM)	820	740
	CoA (0·02 mM)	860	810
	α-Oxoglutarate	920	1050
	α-Oxoglutarate + CoA (0·1 mM)	840	820
	α-Oxoglutarate + CoA (0·02 mM)	920	1920

Table 5. *Oxidation of malate and pyruvate by unshaken and shaken mitochondria*

Incubation medium was as given in Table 3. Concn. of L-malate and pyruvate was 10 mM.

Expt. no.	Additions	q_{O_2} Unshaken mitochondria	q_{O_2} Shaken mitochondria
1	None	520	470
	L-Malate	540	600
	Pyruvate	540	730
	L-Malate + pyruvate	560	840
2	None	660	860
	DPN (0·1 mM)	660	830
	L-Malate	690	1010
	L-Malate + DPN (0·1 mM)	660	1490
3	None	590	570
	DPN (0·1 mM)	570	590
	Pyruvate	560	710
	Pyruvate + DPN (0·1 mM)	610	890

high increase in oxygen uptake with shaken mitochondria.

Electron micrographs. These were prepared from both shaken and unshaken mitochondria.

The mitochondria which were not shaken were oval, or occasionally circular, bodies, 0·6–0·8 μ long and 0·4–0·6 μ wide. The great majority had considerable internal structure and an apparently intact outer membrane. Those mitochondria which were shaken appeared to be the same in size and shape. They had as much internal structure, although a small proportion (10%) had lost their external membrane.

DISCUSSION

Unshaken mitochondria showed comparatively little oxidative activity towards several substrates, and cofactors known to be active in these oxidations usually gave no stimulation of oxygen uptake.

However, if the mitochondria were treated in a way that might be expected to damage them, namely by shaking them in a water bath at 25° for ½ hr., then all the substrates added were oxidized and the cofactors gave large increases in oxygen uptake. It seems likely that in the unshaken mitochondria the enzymes were relatively inaccessible to substrates and cofactors, whereas contact could be made once the mitochondria had been shaken, either because the membrane had become more permeable or because the enzymes had leaked out owing to rupture of the mitochondrial membrane. The electron micrographs showed that some of the mitochondria had lost their membrane, but that most had not, and it is thus probable that both these possibilities are involved. Amoore (1958) has shown that isolated rat-liver mitochondria are permeable to succinate, but relatively impermeable to ATP, and Birt & Bartley (1960) found that DPN did not penetrate. This supports both the apparent inaccessibility of ATP and DPN to the intramitochondrial enzymes observed in this paper and also the oxidation of succinate by unshaken mitochondria.

One cofactor, cytochrome *c*, increased the oxygen uptake with succinate and α-oxoglutarate. This suggests that the unshaken mitochondria were themselves disrupted or permeable to the large cytochrome *c* molecule. However, the cytochrome *c* could be replaced by bovine-plasma albumin, which indicates that the cytochrome *c* was probably acting as a protein, and stabilizing the mitochondria, rather than as a coenzyme.

DPN, when employed at the higher concentration, had a different effect from that of the other cofactors. It might have diffused into the unshaken mitochondria from the high external concentration and thus stimulated the oxidation of α-oxoglutarate, whereas with the shaken mitochondria the higher concentration appears to have caused inhibition.

Many workers (see, for example, Bendall & de Duve, 1960; Greville & Chappell, 1959) have recorded this latency of enzymes, mainly with animal mitochondria. Simon (1958) has observed it with mitochondrial cytochrome oxidase from several plants. For this enzyme he found that incubation at 25° for 1 hr. was quite ineffective in increasing the activity: much more drastic treatment, such as exposure to digitonin or hypotonic conditions, was required. He also compared the manometric and spectrophotometric assays of this enzyme and found that the stimulation of enzyme activity on treatment with digitonin was always less in the manometric assay. It seems possible that this might be due to an increased rate of cytochrome *c* oxidation in the control mitochondria, brought about in a similar way to the stimulations reported for shaken mitochondria in this paper.

SUMMARY

1. A simple polarograph and a cell containing platinum and silver/silver chloride electrodes suitable for measurement of oxygen uptake are described.

2. α-Oxoglutarate, L-malate and pyruvate were either only very slowly oxidized or not oxidized at all by pea-root mitochondria. If the mitochondria were first shaken in a water bath at 25° for ½ hr. they then oxidized these substrates. Succinate was oxidized by the mitochondria, but the rate was increased by shaking.

3. Stimulation of the oxidations by unshaken mitochondria was not obtained by addition of several cofactors (diphosphopyridine nucleotide, adenosine di- and tri-phosphates, thiamine pyrophosphate and coenzyme A) but the oxidations by shaken mitochondria were greatly enhanced.

4. Cytochrome *c* increased the rate of oxidation of succinate and α-oxoglutarate, but could be replaced by bovine-plasma albumin.

5. It is suggested that the action of the shaking is to make the mitochondrial membrane more permeable or to disrupt it and release the enzymes.

I wish to thank Mr D. Bellamy of the Department of Biochemistry, Oxford, and Mr R. T. Garner and Dr R. M. Whittle of this Institution, for much helpful advice and practical assistance during the construction of the polarograph and cell, and Mr J. C. W. Crawley for sectioning and preparing electron micrographs of the mitochondria.

REFERENCES

Amoore, J. E. (1958). *Biochem. J.* **70**, 718.
Bellamy, D. (1958). *Biochem. J.* **70**, 580.
Bellamy, D. & Bartley, W. (1960). *Biochem. J.* **76**, 78.
Bendall, D. S. & de Duve, C. (1960). *Biochem. J.* **74**, 444.
Birt, L. M. & Bartley, W. (1960). *Biochem. J.* **75**, 303.
Greville, G. D. & Chappell, J. B. (1959). *Biochim. biophys. Acta*, **33**, 267.
Simon, E. W. (1958). *Biochem. J.* **69**, 67.
Sunderland, N., Heyes, J. K. & Brown, R. (1957). *J. exp. Bot.* **8**, 55.
Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.

Index of Authors

Note. Pages in *Proceedings of The Biochemical Society* are distinguished by P.

ADAMS, D. H. & BURGESS, E. A. Some factors affecting the permeability of large-granule membranes to mouse-liver catalase *in vitro* 247

ALDRIDGE, W. N., EMERY, R. C. & STREET, B. W. A tissue homogenizer 326

ANDERSON, A. J., LEPPER, M. H. & WINZLER, R. J. The fractionation of urine colloids on anion-exchange cellulose 581

ANDERSON, J. D., HOUGH, L. & PRIDHAM, J. B. The reaction of carbohydrases with phenolic glucosides 564

ANTONINI, E. *see* GIBSON, Q. H. 328

ARNON, D. I., LOSADA, M., NOZAKI, M. & TAGAWA, K. Photofixation of nitrogen and photoproduction of hydrogen by thiosulphate during bacterial photosynthesis 23P

ARNON, R. *see* SELA, M. 394

BACH, S. J. & KILLIP, J. D. The crystallization and properties of hepatic arginase of ox, sheep and horse 7P

BACHRACH, U. Metabolism of amines. 3. The degradation and synthesis of γ-aminobutyric acid by *Pseudomonas aeruginosa* 417

BAIRD, G. D. & PERRY, S. V. The inhibitory action of relaxing-factor preparations on the myofibrillar adenosine triphosphatase 262

BATCHELOR, A. *see* GOLBERG, L. 252

BATT, R. D., DICKENS, F. & WILLIAMSON, D. H. Tetrose metabolism. 1. The preparation and degradation of specifically labelled [^{14}C]tetroses and [^{14}C]tetritols 272

BATT, R. D., DICKENS, F. & WILLIAMSON, D. H. Tetrose metabolism. 2. The utilization of tetroses and tetritols by rat tissues 281

BAULD, W. S. *see* GIVNER, M. L. 400, 406

BELLETT, A. J. D. & BURNESS, A. T. H. Sites of synthesis of a nucleic acid and a protein in infected Krebs-2 ascites tumour cells 17P

BERGMANN, F. & RIMON, S. Fractionation of C-esterase from the hog's kidney extract 203

BIANCHI, P., BUTLER, J. A. V., CRATHORN, A. R. & SHOOTER, K. V. Some characteristics of the phosphorylating kinases of neoplastic and normal cells 15P

BLABER, L. C. & CREASEY, N. H. The mode of recovery of cholinesterase activity *in vivo* after organophosphorus poisoning. 1. Erythrocyte cholinesterase 591

BLABER, L. C. & CREASEY, N. H. The mode of recovery of cholinesterase activity *in vivo* after organophosphorus poisoning. 2. Brain cholinesterase 597

BLAKLEY, R. L. A spectrophotometric study of the reaction catalysed by serine transhydroxymethylase 459

BLASS, J. P. The simple monosubstituted guanidines of mammalian brain 484

BLOXAM, H. R., DAY, M. G., GIBBS, N. K. & WOOLF, L. I. An inborn defect in the metabolism of tyrosine in infants on a normal diet 320

BOND, P. A. *see* FINCHAM, J. R. S. 96

BOOTH, J., BOYLAND, E., SATO, T. & SIMS, P. Metabolism of polycyclic compounds. 17. The reaction of 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene with glutathione catalysed by tissue preparations 182

BOWEN, H. J. M. The determination of tungsten in biological material by activation analysis 79

BOYLAND, E. & SIMS, P. Metabolism of polycyclic compounds. 16. The metabolism of 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene 175

BOYLAND, E. *see also* BOOTH, J. 182

BRIGGS, D. E. Biochemical studies with methanesulphonyl esters, especially β-chloroethyl methanesulphonate 186

BROOKES, P. & LAWLEY, P. D. The reaction of mustard gas with nucleic acids *in vitro* and *in vivo* 478

BROSTOFF, D. V., MOSES, V. & SMITH, M. J. H. Salicylate congeners and uncoupling activity 5P

BUNYAN, J., GREEN, J., EDWIN, E. E. & DIPLOCK, A. T. Studies on vitamin E. 5. Lipid peroxidation in dialuric acid-induced haemolysis of vitamin E-deficient erythrocytes 47

BURDON, R. H. Studies on the biosynthesis of ribonucleic acid 14P

BURGESS, E. A. *see* ADAMS, D. H. 247

BURNESS, A. T. H. *see* BELLETT, A. J. D. 17P

BURT, J. R. Phosphorylation of some analogues of [illegible]ctose by yeast hexokinase 16P

BURTON, K. Frequencies of nucleotide sequences in deoxyribonucleic acids 547

BURTON, K. *see also* FESSLER, L. I. 558

BUSH, I. E. & HOCKADAY, T. D. R. Paper chromatography of some 2:4-dinitrophenylhydrazones in acid systems 7P

BUTLER, J. A. V. *see* BIANCHI, P. 15P; JOHNS, E. W. 631

CAMPBELL, J. W. Pyrimidine metabolism in parasitic flatworms 105

CANELLAKIS, E. S. Pyrophosphorolysis of soluble ribonucleic acid 14P

CANTLEY, M. & HOUGH, L. The periodate oxidation of amino sugars and related compounds 6P

CARPENTER, K. J. The estimation of the available lysine in animal-protein foods 604

CESSI, C. & PILIEGO, F. The determination of amino sugars in the presence of amino acids and glucose 508

CHAPLEN, P. & THOMAS, R. Studies in the biosynthesis of fungal metabolites: the biosynthesis of palitantin 91

COLLINS, J. F. & KORNBERG, H. L. The metabolism of C_2 compounds in micro-organisms. 4. Synthesis of cell materials from acetate by *Aspergillus niger* 430

COOK, R. P. *see* GREIG, M. 16P

COOKE, B. A. & TAYLOR, W. 'Conjugate' formation during the metabolism of [4-^{14}C]progesterone by female-rat liver *in vitro* 21P

COOKE, N. J. *see* HANSEN, R. P. 64

CRATHORN, A. R. *see* BIANCHI, P. 15P

CREASEY, N. H. *see* BLABER, L. C. 591, 597

CREASEY, W. A. The enzymic composition of nuclei isolated from radio-sensitive and non-sensitive tissues with special reference to catalase activity 5

CREETH, J. M. & NICHOL, L. W. Evidence for the chemical interaction of urease in solution 230

CROSBIE, G. W. *see* FLEMING, L. W. 23P

CURZON, G. The effects of some ions and chelating agents on the oxidase activity of caeruloplasmin 66

DAVENPORT, H. E. A protein from leaves catalysing the reduction of metmyoglobin and triphosphopyridine nucleotide by illuminated chloroplasts 471

DAWSON, R. M. C., HEMINGTON, N. & LINDSAY, D. B. The phospholipids of the erythrocyte 'ghosts' of various species 226

DAY, M. G. *see* BLOXAM, H. R. 320
DICKENS, F. & WILLIAMSON, D. H. Metabolism of D- and L-[3-^{14}C]glycerate by liver tissue of the rat 356
DICKENS, F. *see also* BATT, R. D. 272, 281
DICKERSON, J. W. T. *see* WIDDOWSON, E. 30
DIPLOCK, A. T. *see* BUNYAN, J. 47
DODGSON, K. S., POWELL, G. M., ROSE, F. A. & TUDBALL, N. *p*-Hydroxyphenylacetic acid *O*-sulphate: a metabolite of tyrosine *O*-sulphate in the rat 20P
DUNCAN, A. M. *see* DUTTON, G. J. 18P
DUNSTONE, J. R. Ion-exchange reactions between cartilage and various cations 164
DUTTON, G. J. & DUNCAN, A. M. Mechanism of conjugation of *o*-aminophenol in plants and an insect 18P
DUTTON, G. J. *see also* STEVENSON, I. H. 19P

EDWIN, E. E. *see* BUNYAN, J. 47
EMERY, R. C. *see* ALDRIDGE, W. N. 326

FESSLER, L. I. Biosynthesis of bacteriophage deoxyribonucleic acid 16P
FESSLER, L. I., KELEMEN, M. V. & BURTON, K. Synthesis of protein in a purine-requiring *Escherichia coli* infected with bacteriophage T2 558
FEWSON, C. A. & NICHOLAS, D. J. D. Nitrate reduction in *Pseudomonas aeruginosa* 3P
FINCHAM, J. R. S. & BOND, P. A. A further genetic variety of glutamic acid dehydrogenase in *Neurospora crassa* 96
FINDLAY, J. & LEVVY, G. A. Purification of β-*N*-acetylglucosaminidase from the pig epididymis 170
FLEMING, L. W. & CROSBIE, G. W. Non-enzymic transamination between glycine and glyoxylate 23P
FOWDEN, L. *see* NOE, F. F. 543
FRENCH, D. *see* MISRA, U. K. 1P

GAMMACK, D. B. *see* RAPER, A. B. 10P
GERSON, T. The determination of cholesterol and coprosterol in faecal lipids 446
GIBBS, N. K. *see* BLOXAM, H. R. 320
GIBSON, Q. H. The reactions of some aromatic *C*-nitroso compounds with haemoglobin 519
GIBSON, Q. H. & ANTONINI, E. Kinetic studies on the reaction between native globin and haem derivatives 328
GIBSON, Q. H. *see also* MASSEY, V. 341
GIVNER, M. L., BAULD, W. S. & VAGI, K. A chemical method for the quantitative determination of 2-methoxyoestrone, oestrone, ring D α-ketolic oestrogens, oestradiol-17β, 16-*epi*oestriol and oestriol in human urine 406
GIVNER, M. L., BAULD, W. S. & VAGI, K. A method for the quantitative fractionation of mixtures of 2-methoxyoestrone, oestrone, ring D α-ketolic oestrogens, oestradiol-17β, 16-*epi*oestriol and oestriol by partition chromatography and the girard reaction 400
GOLBERG, L., MARTIN, L. E. & BATCHELOR, A. Biochemical changes in the tissues of animals injected with iron: acid phosphatase and other enzymes 252
GOLDBLATT, M. W., HENSON, A. F. & SOMERVILLE, A. R. Metabolism of bladder carcinogens. 3. The metabolic path of 2-[8-^{14}C]naphthylamine in several animal species 511
GOULDING, K. J. *see* NORTHCOTE, D. H. 503
GRAY, G. M. The phospholipids of ox spleen with special reference to the fatty acid and fatty aldehyde compositions of the lecithin and kephalin fractions 82
GRAY, G. M. *see* MACFARLANE, M. G. 626
GREEN, J. *see* BUNYAN, J. 47
GREENBAUM, A. L. *see* WANG, D. Y. 552
GREIG, M. & COOK, R. P. The nature of 'cholesterol' in normal human-blood plasma 16P
GRUNBERG-MANAGO, M. Mode of action of polynucleotide phosphorylase 13P
GUEST, J. R. & WOODS, D. D. Cobalamin and the enzymic formation of a factor concerned in the synthesis of methionine by *Escherichia coli* 422

HAGIWARA, A. *see* PAUL, J. 21P
HALL, R. J. *see* PETERS, R. A. 17
HANSEN, R. P., SHORLAND, F. B. & COOKE, N. J. The isolation of *cis*-Δ^9-heptadecenoic acid from butterfat 64
HARADA, T. *see* SPENCER, B. 305
HARTLEY, B. S. *see* NAUGHTON, M. A. 149
HEALD, P. J. *see* ROSE, S. P. R. 8P
HEATON, F. W. The response to increased magnesium intake 1P
HEMINGTON, N. *see* DAWSON, R. M. C. 226
HENSON, A. F. *see* GOLDBLATT, M. W. 511
HERBERT, E. Effect of phosphodiesterase on the capacity of soluble ribonucleic acid to accept amino acids 14P
HEREDIA, C. F. & MEDINA, A. Nitrate reductase and related enzymes in *Escherichia coli* 24
HIGASHI, T. *see* HORIO, T. 194
HOCKADAY, T. D. R. *see* BUSH, I. E. 7P
HORIO, T., HIGASHI, T., SASAGAWA, M., KUSAI, K., NAKAI, M. & OKUNUKI, K. Preparation of crystalline *Pseudomonas* cytochrome *c*-551 and its general properties 194
HORNE, R. W. *see* NORTHCOTE, D. H. 503
HOUGH, L. *see* ANDERSON, J. D. 564; CANTLEY, M. 6P
HUEHNS, E. R. *see* RAPER, A. B. 10P
HUGHES, R. E. & KODICEK, E. The absence of galactosamine in scorbutic granulation tissue 3P

JEFFREE, G. M. Phosphatase activity in an osteoclastoma 2P
JEFFREE, G. M. Phosphatase activity in bones of hyperparathyroid monkeys 2P
JOCELYN, P. C. The effect of some protein solutions on the oxidation of glutathione in oxygenated erythrocytes 368
JOCELYN, P. C. The reduction of oxidized glutathione in erythrocyte haemolysates in pernicious anaemia 363
JOHANSEN, P. G., MARSHALL, R. D. & NEUBERGER, A. Carbohydrates in protein. 2. The hexose, hexosamine, acetyl and amide-nitrogen content of hen's-egg albumin 239
JOHNS, E. W., PHILLIPS, D. M. P., SIMSON, P. & BUTLER, J. A. V. Improved fractionations of arginine-rich histones from calf thymus 631
JOHNSON, M. K. The intracellular distribution of glycolytic and other enzymes in rat-brain homogenates and mitochondrial preparations 610
JONES, N. R. & MURRAY, J. The acid-soluble nucleotides of codling (*Gadus callarias*) muscle 567

KELEMEN, M. V. *see* FESSLER, L. I. 558
KENT, L. H. & SLADE, J. H. R., Immunochemically-active cross-linked polystyrene preparations 12
KILLIP, J. D. *see* BACH, S. J. 7P
KIMURA, Y. & NAGAI, Y. Infrared spectra of brain phosphatidylserine 3
KIMURA, Y. & NAGAI, Y. Preparation of hydrophilic colloids of brain kephalins 1
KODICEK, E. *see* HUGHES, R. E. 3P
KORNBERG, H. L., PHIZACKERLEY, P. J. R. & SADLER, J. R. The metabolism of C_3 compounds in micro-organisms. 5. Biosynthesis of cell materials from acetate in *Escherichia coli* 438
KORNBERG, H. L. *see also* COLLINS, J. F. 430
KUSAI, K. *see* HORIO, T. 194

LAWLEY, P. D. *see* BROOKES, P. 478
LEPPER, M. H. *see* ANDERSON, A. J. 581
LEVVY, G. A. *see* FINDLAY, J. 170

LINDSAY, D. B. *see* DAWSON, R. M. C. 226
LING, N. R. The attachment of proteins to cells tanned with pyruvic aldehyde 12P
LISI, A. G. *see* SCOTT, D. B. M. 52
LOENING, U. E. Changes in two microsomal components accompanying cell differentiation 20P
LOSADA, M. *see* ARNON, D. I. 23P

MACFARLANE, M. G., GRAY, G. M. & WHEELDON, L. W. Fatty acid composition of phospholipids from subcellular particles of rat liver 626
MACLEOD, L. D. Oxidation of β-phenylethanol by alcohol dehydrogenase of liver 10P
MAGEE, W. L. & THOMPSON, R. H. S. The estimation of phospholipase A activity in aqueous systems 526
MALLINSON, A. C. *see* PROSSER, E. J. T. 24P
MANCHESTER, K. L. & YOUNG, F. G. The influence of the induction of alloxan-diabetes on the incorporation of amino acids into protein of rat diaphragm 386
MARKHAM, R. A graphical method for the rapid determination of sedimentation coefficients 516
MARSHALL, R. D. *see* JOHANSEN, P. G. 239
MARTIN, L. E. *see* GOLBERG, L. 252
MASSEY, V., GIBSON, Q. H. & VEEGER, C. Intermediates in the catalytic action of lipoyl dehydrogenase (diaphorase) 341
MEDINA, A. *see* HEREDIA, C. F. 24
MICHELSON, A. M. Chemical aspects of polynucleotide synthesis 13P
MISRA, U. K. & FRENCH, D. Inhibition and action pattern of β-amylase in presence of maltose 1P
MOSES, V. & SMITH, M. J. H. Anti-inflammatory drugs and intermediary metabolism 5P
MOSES, V. *see also* BROSTOFF, D. V. 5P
MUNRO, H. N. *see* PROSSER, E. J. T. 24P
MURRAY, J. *see* JONES, N. R. 567

NAGAI, Y. *see* KIMURA, Y. 1, 3
NAKAI, M. *see* HORIO, T. 194
NAUGHTON, M. A., SANGER, F., HARTLEY, B. S. & SHAW, D. C. The amino acid sequence around the reactive serine residue of some proteolytic enzymes 149
NEUBERGER, A. *see* JOHANSEN, P. G. 239
NEWTON, B. A. Inhibition of incorporation of ^{14}C-labelled purines into nucleic acids by a substituted pyrimidine 17P
NICHOL, L. W. *see* CREETH, J. M. 230
NICHOLAS, D. J. D. *see* FEWSON, C. A. 3P; WALKER, G. C. 4P
NOE, F. F. & FOWDEN, L. β-Pyrazol-1-ylalanine, an amino acid from water-melon seeds (*Citrullus vulgaris*) 543
NORTHCOTE, D. H., GOULDING, K. J. & HORNE, R. W. The chemical composition and structure of the cell wall of *Hydrodictyon africanum* Yaman 503
NOZAKI, M. *see* ARNON, D. I. 23P

OKUNUKI, K. *see* HORIO, T. 194

PARKE, D. V. Studies in detoxication. 84. The metabolism of [^{14}C]aniline in the rabbit and other animals 493
PARKER, V. H. 1:1:1-Trichloro-2:2-di-(*p*-chlorophenyl)-ethane (DDT) and rat adipose tissue 74
PAUL, J. Micro-determination of soluble silica in urine 202
PAUL, J. & HAGIWARA, A. Inhibitor studies on the time sequence of nucleic acid and protein synthesis in mammalian cells 21P
PAULUS, E. *see* ROUX, D. G. 315
PENNINGTON, R. J. Amino acid-activating enzymes in muscle 205
PERKINS, H. R. *see* ROGERS, H. J. 448
PERRY, S. V. *see* BAIRD, G. D. 262
PETERS, R. A., HALL, R. J., WARD, P. F. V. & SHEPPARD, N. The chemical nature of the toxic compounds containing fluorine in the seeds of *Dichapetalum toxicarium* 17
PHILLIPS, D. M. P. *see* JOHNS, E. W. 631
PHIZACKERLEY, P. J. R. *see* KORNBERG, H. L. 438
PICHAPPA, C. V. *see* SUNDARAM, T. K. 145
PILIEGO, F. *see* CESSI, C. 508
POWELL, G. M. *see* DODGSON, K. S. 20P
PRIDHAM, J. B. *see* ANDERSON, J. D. 564
PROSSER, E. J. T., MALLINSON, A. C. & MUNRO, H. N. The effect of protein intake on the ribonucleic acid of liver cell sap 24P

RAJAGOPALAN, K. V. *see* SUNDARAM, T. K. 145
RANDLE, P. J. *see* VARGAS, L. 43
RAPER, A. B., GAMMACK, D. B., HUEHNS, E. R. & SHOOTER, E. M. Four major haemoglobins in one individual 10P
REID, E. *see* SMITH, C. J. 19P
RICHMOND, M. H. Immunological properties of exopenicillinase synthesized by *Bacillus cereus* 569/H in the presence of amino acid analogues 112
RICHMOND, M. H. Incorporation of DL-β-(*p*-fluorophenyl)-[β-^{14}C]alanine into exopenicillinase by *Bacillus cereus* 569/H 121
RIDD, J. H. & WHITE, R. F. M. Nuclear magnetic resonance spectrum of β-pyrazol-1-ylalanine. *Appendix to*: β-Pyrazol-1-ylalanine, an amino acid from water-melon seeds (*Citrullus vulgaris*) (Noe, F. F. & Fowden, L.) 546
RIMON, S. *see* BERGMANN, F. 209
ROBINSON, D. S. & SALAMAN, M. R. The effect of changes in nutritional state on the clearing-factor lipase activity of homogenates and extracts of rat adipose tissue 8P
ROBINSON, G. B. & SHAW, B. The hydolysis of dipeptides by different regions of rat small intestine 351
ROGERS, H. J. & PERKINS, H. R. 5-Fluorouracil and mucopeptide biosynthesis by *Staphylococcus aureus* 448
ROSE, F. A. *see* DODGSON, K. S. 20P
ROSE, S. P. R. & HEALD, P. J. Phosphoprotein phosphatase in cerebral tissues 8P
ROUX, D. G. & PAULUS, E. Condensed tannins. 6. Biochemical and stereochemical interrelationships of (+)-fustin from black-wattle heartwood (*Acacia mollissima*) 315
ROY, A. B. The sulphatase of ox liver. 7. The intracellular distribution of sulphatases A and B 380

SADLER, J. R. *see* KORNBERG, H. L. 438
SALAMAN, M. R. *see* ROBINSON, D. S. 8P
SANGER, F. *see* NAUGHTON, M. A. 149
SARMA, P. S. *see* SUNDARAM, S. 465; SUNDARAM, T. K. 145
SASAGAWA, M. *see* HORIO, T. 194
SATO, T. *see* BOOTH, J. 182
SCOTT, D. B. M. & LISI, A. G. Changes in enzymes of the uterus of the ovariectomized rat after treatment with oestradiol 52
SCRATCHERD, T. *see* TAYLOR, W. 22P
SCUTT, P. B. Effects of prolonged oxygenation on the cytochrome and enzyme systems of *Torula* 6P
SELA, M. & ARNON, R. Studies on the chemical basis of the antigenicity of proteins. 3. The role of rigidity in the antigenicity of polypeptidyl gelatins 394
SENTHESHANMUGANATHAN, S. The purification and properties of the tyrosine–2-oxoglutarate transaminase of *Saccharomyces cerevisiae* 619
SHAW, B. *see* ROBINSON, G. B. 351
SHAW, D. C. *see* NAUGHTON, M. A. 149
SHEPPARD, N. Notes on the nuclear-magnetic-resonance spectrum of ω-fluorodecanoic acid and of a fluorooctadecenoic acid of unknown structure. *Appendix to*: The chemical nature of the toxic compounds containing fluorine in the seeds of *Dichapetalum toxicarium* (Peters, R. A., Hall, R. J., Ward, P. F. V. & Sheppard, N.) 22

SHEPPARD, N. *see also* PETERS, R. A. 17
SHOOTER, E. M. *see* RAPER, A. B. 10P
SHOOTER, K. V. *see* BIANCHI, P. 15P
SHORLAND, F. B. *see* HANSEN, R. P. 64
SIMKIN, J. L. & SUTTON, C. R. The incorporation of radioactive amino acids into proteins of the microsome fraction of guinea-pig liver at very short time intervals after administration 489
SIMS, P. *see* BOOTH, J. 182; BOYLAND, E. 175
SIMSON, P. *see* JOHNS, E. W. 631
SLADE, J. H. R. *see* KENT, L. H. 12
SMELLIE, R. M. S. Studies on the biosynthesis of deoxyribonucleic acid 15P
SMITH, C. J. & REID, E. Transfer of soluble ribonucleic acid to microsomes in an *in vitro* system from rat liver 19P
SMITH, M. J. H. *see* BROSTOFF, D. V. 5P; MOSES, V. 5P
SOMERVILLE, A. R. *see* GOLDBLATT, M. W. 511
SPENCER, B. Endogenous sulphate acceptors in rat liver 294
SPENCER, B. & HARADA, T. The role of choline sulphate in the sulphur metabolism of fungi 305
STENESH, J. J. & WINNICK, T. Carnosine–anserine synthetase of muscle. 4. Partial purification of the enzyme and further studies of β-alanyl peptide synthesis 575
STEVENSON, I. H. & DUTTON, G. J. Mechanism of glucuronide synthesis in skin 19P
STICKLAND, R. G. Polarographic measurement of oxygen uptake by pea-root mitochondria 636
STREET, B. W. *see* ALDRIDGE, W. N. 326
SUNDARAM, S. & SARMA, P. S. Purification and properties of a protease from the gut of *Etroplus suratensis* 465
SUNDARAM, T. K., RAJAGOPALAN, K. V., PICHAPPA, C. V. & SARMA, P. S. Studies on pyridine nucleotide biosynthesis 145
SUTTON, C. R. *see* SIMKIN, J. L. 489

TAGAWA, K. *see* ARNON, D. I. 23P
TATA, J. R. The partial purification and properties of thyroxine dehalogenase 214
TAYLOR, K. W. *see* VARGAS, L. 43
TAYLOR, W. & SCRATCHERD, T. Progesterone metabolism in the cat 22P
TAYLOR, W. *see also* COOKE, B. A. 21P
THOMAS, R. *see* CHAPLEN, P. 508
THOMPSON, R. H. S. *see* MAGEE, W. L. 526
TUDBALL, N. *see* DODGSON, K. S. 20P

VAGI, K. *see* GIVNER, M. L. 400, 406
VARGAS, L., TAYLOR, K. W. & RANDLE, P. J. Insulin and an inhibitor of glucose uptake, in protein fractions of normal human plasma 43
VEEGER, C. *see* MASSEY, V. 341

WADE, H. E. The fractionation of phosphate esters on ion-exchange resin by a new system of pH-gradient elution 534
WALKER, G. C. & NICHOLAS, D. J. D. Nitrite reductase from *Pseudomonas aeruginosa* 4P
WANG, D. Y. & GREENBAUM, A. L. Levels and distribution of the acid-soluble ribonucleotides in the mammary glands of pregnant and lactating rats 552
WARD, P. F. V. *see* PETERS, R. A. 17
WESTALL, R. G. Argininosuccinic aciduria: identification and reactions of the abnormal metabolite in a newly described form of mental disease, with some preliminary metabolic studies 135
WHEELDON, L. W. *see* MACFARLANE, M. G. 626
WHITE, R. F. M. *see* RIDD, J. H. 546
WIDDOWSON, E. M. & DICKERSON, J. W. T. The effect of growth and function on the chemical composition of soft tissues 30
WILLIAMSON, D. H. *see* BATT, R. D. 272, 281; DICKENS, F. 356
WINNICK, T. *see* STENESH, J. J. 575
WINZLER, R. J. *see* ANDERSON, A. J. 581
WOODS, D. D. *see* GUEST, J. R. 422
WOLFE, L. S. The subcellular distribution of gangliosides in cerebral cortex 9P
WOOLF, L. I. *see* BLOXAM, H. R. 320

YOUNG, F. G. *see* MANCHESTER K. L. 386

ZELITCH I. Synthesis of glycollic acid by spinach chloroplasts 11P

Index of Subjects

Note. Pages in *Proceedings of The Biochemical Society* are distinguished by P.

Acacia mollissima, biochemical and stereochemical interrelationships of (+)-fustin from (Roux, D. G. & Paulus E.) 315

Acetate, biosynthesis of cell materials from, in *Escherichia coli* (Kornberg, H. L., Phizackerley, P. J. R. & Sadler, J. R.) 438

[1-^{14}C]Acetate, incorporation of, into palitantin (Chaplen, P. & Thomas, R.) 91

Acetate, synthesis of cell materials from, by *Aspergillus niger* (Collins, J. F. & Kornberg, H. L.) 430

Acetyl content of hen's-egg albumin (Johansen, P. G., Marshall, R. D. & Neuberger, A.) 239

β-*N*-Acetylglucosaminidase, purification of, from pig epididymis (Findlay, J. & Levvy, G. A.) 170

Acid phosphatase in tissues of animals injected with iron (Golberg, L., Martin, L. E. & Batchelor, A.) 252

Actin sulphate in rat liver (Spencer, B.) 294

Adenosine 3′-phosphate 5′-sulphatophosphate in rat liver (Spencer, B.) 294

Adenosine 3′-phosphate 5′-sulphatophosphate in fungi (Spencer, B. & Harada, T.) 305

Adenosine 5′-sulphatophosphate in rat liver (Spencer, B.) 249

Adenosine triphosphatase, myofibrillar, inhibitory action of relaxing-factor preparations on (Baird, G. D. & Perry, S. V.) 262

Adipose tissue, rat, and 1:1:1-trichloro-2:2-di-(*p*-chlorophenyl)ethane (DDT) (Parker, V. H.) 74

Adipose tissue, rat, effect of changes in nutritional state on clearing-factor lipase activity of homogenates and extracts of (Robinson, D. S. & Salaman, M R.) 8P

β-Alanine, production of, by rat tapeworm (Campbell, J. W.) 105

β-Alanyl peptide, synthesis of (Stenesh, J. J. & Winnick, T.) 575

Albumin, hen's-egg, hexose, hexosamine, acetyl and amide-nitrogen content of (Johansen, P. G., Marshall, R. D. & Neuberger, A.) 239

Alcohol dehydrogenase of liver, oxidation of β-phenylethanol by (MacLeod, L. D.) 10P

7-Alkylguanine, synthesis of (Brookes, P. & Lawley, P. D.) 478

Alloxan-diabetes, influence of, on incorporation of amino acids into protein of rat diaphragm (Manchester, K. L. & Young, F. G.) 386

Amide-nitrogen content of hen's-egg albumin (Johansen, P. G., Marshall, R. D. & Neuberger, A.) 239

Amino acid analogues, immunological properties of exopenicillinase synthesized by *Bacillus cereus* 569/H in the presence of (Richmond, M. H.) 112

Amino acid sequence around the reactive serine residue of some proteolytic enzymes (Naughton, M. A., Sanger, F., Hartley, B. S. & Shaw, D. C.) 149

Amino acid-activating enzymes in muscle (Pennington, R. J.) 205

Amino acids, determination of amino sugars in the presence of (Cessi, C. & Piliego, F.) 508

Amino acids, effect of phosphodiesterase on capacity of soluble ribonucleic acid to accept (Herbert, E.) 14P

Amino acids, influence of alloxan-diabetes on incorporation of, into protein of rat diaphragm (Manchester, K. L. & Young, F. G.) 386

Amino acids, radioactive, incorporation of, into proteins of microsome fraction of guinea-pig liver (Simkin, J. L. & Sutton, C. R.) 489

γ-Aminobutyric acid, degradation and synthesis of, by *Pseudomonas aeruginosa* (Bachrach, U.) 417

β-Amino*iso*butyric acid, production of, by rat tapeworm (Campbell, J. W.) 105

2-Amino-1-naphthyl hydrogen sulphate as metabolite of naphthylamine (Goldblatt, M. W., Henson, A. F. & Somerville, A. R.) 511

Aminophenol, excretion of, in rabbits (Parke, D. V.) 493

o-Aminophenol, mechanism of conjugation of, in plants and an insect (Dutton, G. J. & Duncan, A. M.) 18P

Amino sugars, a distillation method for extraction of (Cessi, C. & Piliego, F.) 508

Amino sugars and related compounds, periodate oxidation of (Cantley, M. & Hough, L.) 6P

Amino sugars, determination of, in the presence of amino acids and glucose (Cessi, C. & Piliego, F.) 508

β-Amylase, inhibition and action pattern of, in the presence of maltose (Misra, U. K. & French, D.) 1P

[^{14}C]Aniline, metabolism of, in animals (Parke, D. V.) 493

Animal-protein foods, estimation of available lysine in (Carpenter, K. J.) 604

Antigens, role of rigidity in function of polypeptidyl gelatins as (Sela, M. & Arnon, R.) 394

Anti-inflammatory drugs and intermediary metabolism (Moses, V. & Smith, M. J. H.) 5P

Arbutin, formation of *p*-hydroxyphenyl β-gentiobioside from (Anderson, J. D., Hough, L. & Pridham, J. B.) 564

Arginine of mammalian brain (Blass, J. P.) 484

Arginine-rich histones, improved fractionations of, from calf thymus (Johns, E. W., Phillips, D. M. P., Simpson, P. & Butler, J. A. V.) 631

Argininosuccinic acid, formation of cyclic anhydrides in aqueous solution of (Westall, R. G.) 135

Argininosuccinic aciduria (Westall, R. G.) 135

Ascites-tumour cells, infected Krebs-2, sites of synthesis of a nucleic acid and a protein in (Bellett, A. J. D. & Burness, A. T. H.) 17P

Ascites tumour, Ehrlich, reaction *in vitro* and *in vivo* of nucleic acids of, with mustard gas (Brookes, P. & Lawley, P. D.) 478

Aspartyl peptides, interconversion of α- and β-forms during hydrolysis of (Naughton, M. A., Sanger, F., Hartley, B. S. & Shaw, D. C.) 149

Aspergillus niger, synthesis of cell materials from acetate by (Collins, J. F. & Kornberg, H. L.) 430

Bacillus cereus 569/H, immunological properties of exopenicillinase synthesized by, in the presence of amino acid analogues (Richmond, M. H.) 112

Bacillus cereus 569/H, incorporation of DL-β-(*p*-fluorophenyl)[β-^{14}C]alanine into exopenicillinase by (Richmond, M. H.) 121

Bacillus megaterium, reaction *in vitro* and *in vivo* of nucleic acids of, with mustard gas (Brookes, P. & Lawley, P. D.) 478

Bacteriophage deoxyribonucleic acid, biosynthesis of (Fessler, L. I.) 16P

Bacteriophage T2, synthesis of protein in a purine-requiring *Escherichia coli* infected with (Fessler, L. I., Kelemen, M. V. & Burton, K.) 558

Black-wattle heartwood, biochemical and stereochemical interrelationships of (+)-fustin from (Roux, D. G. & Paulus, E.) 315

Bones of hyperparathyroid monkeys, phosphatase activity in (Jeffree, G. M.) 2P
Brain cholinesterase, mode of recovery *in vivo* of, after organophosphorus poisoning (Blaber, L. C. & Creasey, N. H.) 597
Brain, effect of growth on chemical composition of (Widdowson, E. M. & Dickerson, J. W. T.) 30
Brain kephalins, preparation of hydrophilic colloids of (Kimura, Y. & Nagai, Y.) 1
Brain, mammalian, simple monosubstituted guanidines of (Blass, J. P.) 484
Brain phosphatidylserine, infrared spectra of (Kimura, Y. & Nagai, Y.) 3
Brain, rat, intracellular distribution of glycolytic enzymes in homogenates and mitochondrial preparations from (Johnson, M. K.) 610
Butterfat, isolation of *cis*-Δ^9-heptadecenoic acid from (Hansen, R. P., Shorland, F. B. & Cooke, N. J.) 64

Caeruloplasmin, effects of ions and chelating agents on oxidase activity of (Curzon, G.) 66
Carbohydrases, reaction of, with phenolic glucosides (Anderson, J. D., Hough, L. & Pridham, J. B.) 564
Cardiac muscle, effect of growth on chemical composition of (Widdowson, E. M. & Dickerson, J. W. T.) 30
Carnosine–anserine synthetase of muscle, partial purification of (Stenesh, J. J. & Winnick, T.) 575
Cartilage, ion-exchange reactions between cations and (Dunstone, J. R.) 164
Catalase activity, enzymic composition of nuclei isolated from radio-sensitive and non-sensitive tissues with special reference to (Creasey, W. A.) 5
Cations, ion-exchange reactions between cartilage and (Dunstone, J. R.) 164
Cell differentiation, changes in two microsomal components accompanying (Loening, U. E.) 20P
Cell materials, biosynthesis of, from acetate in *Escherichia coli* (Kornberg, H. L., Phizackerley, P. J. R. & Sadler, J. R.) 438
Cell materials, synthesis of, from acetate by *Aspergillus niger* (Collins, J. F. & Kornberg, H. L.) 430
Cellulose, anion-exchange, fractionation of urine colloids on (Anderson, A. J., Leppers, M. H. & Winzler, R. J.) 581
Cell wall of *Hydrodictyon africanum*, chemical composition and structure of (Northcote, D. H., Goulding, K. J. & Horne, R. W.) 503
Cells, mammalian, inhibitor studies on time sequence of nucleic acid and protein synthesis in (Paul, J. & Hagiwara, A.) 21P
Cells, normal, some characteristics of phosphorylating kinases of (Bianchi, P., Butler, J. A. V., Crathorn, A. R. & Shooter, K. V.) 15P
Cerebral cortex, subcellular distribution of gangliosides in (Wolfe, L. S.) 9P
Cerebral tissues, phosphoprotein phosphatase in (Rose, S. P. R. & Heald, P. J.) 8P
Chelating agents, effects of ions and, on oxidase activity of caeruloplasmin (Curzon, G.) 66
β-Chloroethyl methanesulphonate, biochemical studies with (Briggs, D. E.) 186
Chloroplasts, illuminated, protein from leaves catalysing reduction of metmyoglobin and triphosphopyridine nucleotide by (Davenport, H. E.) 471
Chloroplasts, spinach, synthesis of glycollic acid by (Zelitch, I.) 11P
Cholesterol, determination of, in faecal lipids (Gerson, T.) 446
'Cholesterol', nature of, in normal human-blood plasma (Greig, M. & Cook, R. P.) 16P
Cholinesterase, brain, mode of recovery *in vivo* of, after organophosphorus poisoning (Blaber, L. C. & Creasey, N. H.) 597
Cholinesterase, erythrocyte, mode of recovery *in vivo* of, after organophosphorus poisoning (Blaber, L. C. & Creasey, N. H.) 591
Choline sulphate, role of, in sulphur metabolism of fungi (Spencer, B. & Harada, T.) 305
Choline sulphokinase in fungi (Spencer, B. & Harada, T.) 305
Chondroitin sulphate in fungi (Spencer, B. & Harada, T.) 305
Chymotrypsin, di*iso*propylphosphoryl derivatives of (Naughton, M. A., Sanger, F., Hartley, B. S. & Shaw, D. C.) 149
*iso*Citratase of *Aspergillus niger* (Collins, J. F. & Kornberg, H. L.) 430
*iso*Citratase of *Escherichia coli* (Kornberg, H. L., Phizackerley, P. J. R. & Sadler, J. R.) 438
Citrullus vulgaris, *see* Water melon
Cobalamin and enzymic formation of a factor concerned in synthesis of methionine by *Escherichia coli* (Guest, J. R. & Woods, D. D.) 422
Codling muscle, acid-soluble nucleotides of (Jones, N. R. & Murray, J.) 567
Colloids, hydrophilic, of brain kephalins, preparation of (Kimura, Y. & Nagai, Y.) 1
Coprosterol, determination of, in faecal lipids (Gerson, T.) 446
Cytochrome, effects of prolonged oxygenation on enzyme systems and, of *Torula* (Scutt, P. B.) 6P
Cytochrome *c*-551, *Pseudomonas*, crystalline, preparation and general properties of (Horio, T., Higashi, T., Sasagawa, M., Kusai, K., Nakai, M. & Okunukki, K.) 194

DDT *see* 1:1:1-Trichloro-2:2-di-(*p*-chlorophenyl)ethane
Deoxyribonucleic acid, bacteriophage, biosynthesis of (Fessler, L. I.) 16P
Deoxyribonucleic acid, biosynthesis of (Smellie, R. M. S.) 15P
Deoxyribonucleic acids, frequencies of nucleotide sequences in (Burton, K.) 547
Dialuric acid-induced haemolysis of vitamin E-deficient erythrocytes, lipid peroxidation in (Bunyan, J., Green, J., Edwin, E. E. & Diplock, A. T.) 47
Diaphorase (lipoyl dehydrogenase), intermediates in catalytic action of (Massey, V., Gibson, Q. H. & Veeger, C.) 341
Diaphragm, rat, influence of alloxan-diabetes on incorporation of amino acids into protein of (Manchester, K. L. & Young, F. G.) 386
Dichapetalum toxicarium, chemical nature of toxic compounds containing fluorine in seeds of (Peters, R. A., Hall, R. J., Ward, P. F. V. & Sheppard, N.) 17
1:2-Dihydronaphthalene, metabolism of (Boyland, E. & Sims, P.) 175
1:2-Dihydronaphthalene, reaction of, with glutathione catalysed by tissue preparations (Booth, J., Boyland, E., Sato, T. & Sims, P.) 182
Dihydroxy[^{14}C]acetone, enzymic phosphorylation of (Batt, R. D., Dickens, F. & Williamson, D. H.) 281
2:4-Dinitrophenylhydrazones, paper chromatography of, in acid systems (Bush, I. E. & Hockaday, T. D. R.) 7P
Dipeptides, hydrolysis of, by different regions of rat small intestine (Robinson, G. B. & Shaw, B.) 351

Egg albumin, content of hexose, hexosamine, acetyl and amide-nitrogen (Johansen, P. G., Marshall, R. D. & Neuberger, A.) 239
Elastase, di*iso*propoxy[^{32}P]phosphinyl derivatives of (Naughton, M. A., Sanger, F., Hartley, B. S. & Shaw, D. C.) 149
Epididymis, pig, purification of β-*N*-acetylglucosaminidase from (Findlay, J. & Levvy, G. A.) 170
1:2-Epoxy-1:2:3:4-tetrahydronaphthalene, metabolism of (Boyland, E. & Sims, P.) 175

1:2-Epoxy-1:2:3:4-tetrahydronaphthalene, reaction of, with glutathione catalysed by tissue preparations (Booth, J., Boyland, E., Sato, T. & Sims, P.) 182
Erythrocyte cholinesterase, mode of recovery *in vivo* of, after organophosphorus poisoning (Blaber, L. C. & Creasey, N. H.) 591
Erythrocyte 'ghosts', phospholipids of (Dawson, R. M. C., Hemington, N. & Lindsay, D. B.) 226
Erythrocyte haemolysates in pernicious anaemia, reduction of oxidized glutathione in (Jocelyn, P. C.) 363
Erythrocytes, oxygenated, effect of protein solutions on oxidation of glutathione in (Jocelyn, P. C.) 368
Erythrocytes, vitamin E-deficient, lipid peroxidation in dialuric acid-induced haemolysis of (Bunyan, J., Green, J., Edwin, E. E. & Diplock, A. T.) 47
D-[4-^{14}C]Erythrose, preparation of (Batt, R. D., Dickens, F. & Williamson, D. H.) 272
L-Erythrulose, preparation of specifically labelled (Batt, R. D., Dickens, F. & Williamson, D. H.) 272
Escherichia coli, biosynthesis of cell materials from acetate in (Kornberg, H. L., Phizackerley, P. J. R. & Sadler, J. R.) 438
Escherichia coli, cobalamin and enzymic formation of a factor concerned in synthesis of methionine by (Guest, J. R. & Woods, D. D.) 422
Escherichia coli infected with bacteriophage T2, purine-requiring, synthesis of protein in a (Fessler, L. I., Kelemen, M. V. & Burton, K.) 558
Escherichia coli, nitrate reductase and related enzymes in (Heredia, C. F. & Medina, A.) 24
C-Esterase, fractionation of, from hog-kidney extract (Bergmann, F. & Rimon, S.) 209
Etroplus suratensis, purification and properties of a protease from gut of (Sundaram, S. & Sarma, P. S.) 465
Exopenicillinase, immunological properties of, synthesized by *Bacillus cereus* 569/H in the presence of amino acid analogues (Richmond, M. H.) 112
Exopenicillinase, incorporation of DL-β-(*p*-fluorophenyl)-[β-^{14}C]alanine into, by *Bacillus cereus* 569/H (Richmond, M. H.) 121

Fatty acid composition of lecithin and kephalin fractions of ox-spleen phospholipids (Gray, G. M.) 82
Fatty acid composition of phospholipids from subcellular particles of rat liver (Macfarlane, M. G., Gray, G. M. & Wheeldon, L. W.) 626
Fatty aldehyde composition of lecithin and kephalin fractions of ox-spleen phospholipids (Gray, G. M.) 82
Fisetin, radial distribution of, in wattle heartwood (Roux, D. G. & Paulus, E.) 315
Flatworms, parasitic, pyrimidine metabolism in (Campbell, J. W.) 105
Fluorine, chemical nature of toxic compounds containing, in seeds of *Dichapetalum toxicarium* (Peters, R. A., Hall, R. J., Ward, P. F. V. & Sheppard, N.) 17
ω-Fluorodecanoic acid, nuclear-magnetic-resonance spectrum of (Sheppard, N.) 22
Fluoro-octadecenoic acid of unknown structure, nuclear-magnetic-resonance spectrum of (Sheppard, N.) 22
ω-Fluoro-oleic acid, isolation of, from seeds of *Dichapetalum toxicarium* (Peters, R. A., Hall, R. J., Ward, P. F. V. & Sheppard, N.) 17
DL-β-(*p*-Fluorophenyl)[β-^{14}C]alanine, incorporation of, into exopenicillinase by *Bacillus cereus* 569/H (Richmond, M. H.) 121
5-Fluorouracil biosynthesis by *Staphylococcus aureus* (Rogers, H. J. & Perkins, H. R.) 448
Fructose, phosphorylation by yeast hexokinase of some analogues of (Burt, J. R.) 16P
Fungi, role of choline sulphate in sulphur metabolism of (Spencer, B. & Harada, T.) 305
(+)-Fustin from black-wattle heartwood, biochemical and stereochemical interrelationships of (Roux, D. G. & Paulus, E.) 315

Gadus callarias, *see* Codling
Galactosamine, absence of, in scorbutic granulation tissue (Hughes, R. E. & Kodicek, E.) 3P
Gangliosides, subcellular distribution of, in cerebral cortex (Wolfe, L. S.) 9P
Globin and haem derivatives, kinetic studies on reaction between (Gibson, Q. H. & Antonini, E.) 328
Glucosamine, presence of, in hen's-egg albumin (Johansen, P. G., Marshall, R. D. & Neuberger, A.) 239
Glucose, determination of amino sugars in the presence of (Cessi, C. & Piliego, F.) 508
Glucose in cell wall of *Hydrodictyon africanum* (Northcote, D. H., Goulding, K. J. & Horne, R. W.) 503
Glucose 6-phosphate dehydrogenase, activity of, in rat uterus (Scott, D. B. M. & Lisi, A. G.) 52
Glucose uptake, insulin and an inhibitor of, in protein fractions of normal human plasma (Vargas, L., Taylor, K. W. & Randle, P. J.) 43
Glucosides, phenolic, reaction of carbohydrases with (Anderson, J. D., Hough, L. & Pridham, J. B.) 564
Glucuronide synthesis in skin, mechanism of (Stevenson, I. H. & Dutton, G. J.) 19P
Glutamic acid dehydrogenase, further genetic variety of, in *Neurospora crassa* (Fincham, J. R. S. & Bond, P. A.) 96
Glutathione in oxygenated erythrocytes, effect of protein solutions on oxidation of (Jocelyn, P. C.) 368
Glutathione, oxidized, reduction of, in erythrocyte haemolysates in pernicious anaemia (Jocelyn, P. C.) 363
Glutathione, reaction of 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene with, catalysed by tissue preparations (Booth, J., Boyland, E., Sato, T. & Sims, P.) 182
D- and L-[3-^{14}C]Glycerate, metabolism of, by liver tissue of rat (Dickens, F. & Williamson, D. H.) 356
Glycine and glyoxylate, non-enzymic transamination between (Fleming, L. W. & Crosbie, G. W.) 23P
Glycocyamine of mammalian brain (Blass, J. P.) 484
Glycogen, distribution of ^{14}C incorporated from specifically labelled tetroses and tetritols in (Batt R. D., Dickens, F. & Williamson, D. H.) 281
[2-^{14}C]Glycolaldehyde, preparation of (Batt, R. D., Dickens, F. & Williamson, D. H.) 272
Glycollic acid, synthesis of, by spinach chloroplasts (Zelitch, I.) 11P
Glycolytic enzymes, intracellular distribution of, in rat-brain homogenates and mitochondrial preparations (Johnson, M. K.) 610
Glyoxylate and glycine, non-enzymic transamination between (Fleming, L. W. & Crosbie, G. W.) 23P
Growth, effect of, on chemical composition of soft tissues (Widdowson, E. M. & Dickerson, J. W. T.) 30
Guanidines, simple monosubstituted, of mammalian brain (Blass, J. P.) 484
γ-Guanidobutyric acid of mammalian brain (Blass, J. P.) 484
Guanine, reaction of, with mustard gas (Brookes, P. & Lawley, P. D.) 478
Guanosine, reaction of, with mustard gas (Brookes, P. & Lawley, P. D.) 478

Haem derivatives, kinetic studies on reaction between native globin and (Gibson, Q. H. & Antonini, E.) 328
Haemoglobin, reactions of aromatic *C*-nitroso compounds with (Gibson, Q. H.) 519
Haemoglobins, major, four, identification of, in one individual (Raper, A. B., Gammack, D. B., Huehns, E. R. & Shooter, E. M.) 10P
Hepatic arginase, crystallization and properties of, of ox, sheep and horse (Bach, S. J. & Killip, J. D.) 7P

cis-Δ^9-Heptadecenoic acid, isolation of, from butterfat (Hansen, R. P., Shorland, F. B. & Cooke, N. J.) 64
Hexokinase, activity of, in rat uterus (Scott, D. B. M. & Lisi, A. G.) 52
Hexokinase, yeast, phosphorylation of some analogues of fructose by (Burt, J. R.) 16P
Hexosamine content of hen's-egg albumin (Johansen, P. G., Marshall, R. D. & Neuberger, A.) 239
Hexose content of hen's-egg albumin (Johansen, P. G., Marshall, R. D. & Neuberger, A.) 239
Histones, arginine-rich, improved fractionations of, from calf thymus (Johns, E. W., Phillips, D. M. P., Simpson, P. & Butler, J. A. V.) 631
Homogenizer, tissue (Aldridge, W. N., Emery, R. C. & Street, B. W.) 326
Hydrodictyon africanum, chemical composition and structure of cell wall of (Northcote, D. H., Goulding, K. J. & Horne, R. W.) 503
Hydrogen, photoproduction of, by thiosulphate during bacterial photosynthesis (Arnon, D. I., Losada, M., Nozaki, M. & Tagawa, K.) 23P
o-Hydroxybenzyl β-glucopyranoside, synthesis of, from salicin by action of β-glucosidase (Anderson, J. D., Hough, L. & Pridham, J. B.) 564
7-(β-Hydroxyethylthioethyl)guanine, synthesis of (Brookes, P. & Lawley, P. D.) 478
16α-Hydroxyoestrone, quantitative fractionation of mixtures of, with other oestrogens (Givner, M. L., Bauld, W. S. & Vagi, K.) 400
p-Hydroxyphenylacetic acid, excretion of, in urine by infants (Bloxam, H. R., Day, M. G., Gibbs, N. K. & Woolf, L. I.) 320
p-Hydroxyphenylacetic acid *O*-sulphate as a metabolite of tyrosine *O*-sulphate in the rat (Dodgson, K. S., Powell, G. M., Rose, F. A. & Tudball, N.) 20P
p-Hydroxyphenyl β-gentiobioside, synthesis of, from arbutin by action of β-glucosidase (Anderson, J. D., Hough, L. & Pridham, J. B.) 564
p-Hydroxyphenyl-lactic acid, excretion of, in urine by infants (Bloxam, H. R., Day, M. G., Gibbs, N. K. & Woolf, L. I.) 320
p-Hydroxyphenylpyruvic acid, excretion of, in urine by infants (Bloxam, H. R., Day, M. G., Gibbs, N. K. & Woolf, L. I.) 320
Hydroxy[3-^{14}C]pyruvate, preparation of (Batt, R. D., Dickens, F. & Williamson, D. H.) 272

Infrared spectra of brain phosphatidylserine (Kimura, Y. & Nagai, Y.) 3
Insulin and an inhibitor of glucose uptake, in protein fractions of normal human plasma (Vargas, L., Taylor, K. W. & Randle, P. J.) 43
Insulin, plasma content of (Manchester, K. L. & Young, F. G.) 386
Ion-exchange reactions between cartilage and cations (Dunstone, J. R.) 164
Ions, effects of chelating agents and, on oxidase activity of caeruloplasmin (Curzon, G.) 66
Intestine, small, rat, hydrolysis of dipeptides by different regions of (Robinson, G. B. & Shaw, B.) 351
Iron, acid phosphatase in tissues of animals injected with (Golberg, L., Martin, L. E. & Batchelor, A.) 252

Kephalin and lecithin fractions, phospholipids of ox spleen with special reference to the fatty acid and fatty aldehyde compositions of (Gray, G. M.) 82
Kephalins, brain, preparation of hydrophilic colloids of (Kimura, Y. & Nagai, Y.) 1
Kidney, effect of growth on chemical composition of (Widdowson, E. M. & Dickerson, J. W. T.) 30
Kidney extract, hog, fractionation of C-esterase from (Bergmann, F. & Rimon, S.) 209
Kinases, phosphorylating, of neoplastic and normal cells, some characteristics of (Bianchi, P., Butler, J. A. V., Crathorn, A. R. & Shooter, K. V.) 15P

Lecithin and kephalin fractions, phospholipids of ox spleen with special reference to the fatty acid and fatty aldehyde compositions of (Gray, G. M.) 82
Leuconostoc mesenteroides, metabolism of nicotinic acid and nicotinamide by (Sundaram, T. K., Rajagopalan, K. V., Pichappa, C. V. & Sarma, P. S.) 145
Lipase activity, clearing-factor, effect of changes in nutritional state on, of homogenates and extracts of rat adipose tissue (Robinson, D. S. & Salaman, M. R.) 8P
Lipid peroxidation in dialuric acid-induced haemolysis of vitamin E-deficient erythrocytes (Bunyan, J., Green, J., Edwin, E. E. & Diplock, A. T.) 47
Lipids, faecal, determination of cholesterol and coprosterol in (Gerson, T.) 446
Lipoyl dehydrogenase (diaphorase), intermediates in catalytic action of (Massey, V., Gibson, Q. H. & Veeger, C.) 341
Liver catalase, mouse, permeability of large-granule membranes to, *in vitro* (Adams, D. H. & Burgess, E. A.) 247
Liver-cell sap, effect of protein intake on ribonucleic acid of (Prosser, E. J. T., Mallinson, A. C. & Munro, H. N.) 24P
Liver, effect of growth on chemical composition of (Widdowson, E. M. & Dickerson, J. W. T.) 30
Liver, female rat, 'conjugate' formation during metabolism of [4-^{14}C]progesterone by (Cooke, B. A. & Taylor, W.) 21P
Liver, guinea-pig, microsome fraction of, incorporation of radioactive amino acids into proteins of (Simkin, J. L. & Sutton, C. R.) 489
Liver, oxidation of β-phenylethanol by alcohol dehydrogenase of (MacLeod, L. D.) 10P
Liver, rat, endogenous sulphate acceptors in (Spencer, B.) 294
Liver, rat, fatty acid composition of phospholipids from subcellular particles of (Macfarlane, M. G., Gray, G. M. & Wheeldon, L. W.) 626
Liver, rat, transfer of soluble ribonucleic acid to microsomes in a system *in vitro* from (Smith, C. J. & Reid, E.) 19P
Liver tissue of rat, metabolism of D- and L-[3-^{14}C]glycerate by (Dickens, F. & Williamson, D. H.) 356
Lysine, available, estimation of, in animal-protein foods (Carpenter, K. J.) 604

Magnesium intake, increased, response to (Heaton, F. W.) 1P
Malate synthetase of *Aspergillus niger* (Collins, J. F. & Kornberg, J. F.) 430
Malate synthetase of *Escherichia coli* (Kornberg, H. L., Phizackerley, P. J. R. & Sadler, J. R.) 438
Maltose, inhibition and action pattern of β-amylase in the presence of (Misra, U. K. & French, D.) 1P
Mammary glands of pregnant and lactating rats, levels and distribution of acid-soluble ribonucleotides in (Wang, D. Y. & Greenbaum, A. L.) 552
Mannose in cell wall of *Hydrodictyon africanum* (Northcote, D. H., Goulding, K. J. & Horne, R. W.) 503
Mannose, presence of, in hen's-egg albumin (Johansen, P. G., Marshall, R. D. & Neuberger, A.) 239
Membranes, large-granule, permeability of, to mouse-liver catalase *in vitro* (Adams, D. H. & Burgess, E. A.) 247
Methanesulphonyl esters, biochemical studies with (Briggs, D. E.) 186
Methionine, cobalamin and enzymic formation of a factor concerned in synthesis of, by *Escherichia coli* (Guest, J. R. & Woods, D. D.) 422
2-Methoxyoestrone, quantitative determination of, in human urine (Givner, M. L., Bauld, W. S. & Vagi, K.) 406
2-Methoxyoestrone, quantitative fractionation of mixtures of, with other oestrogens (Givner, M. L., Bauld, W. S. & Vagi, K.) 400

Metmyoglobin, protein from leaves catalysing reduction of, by illuminated chloroplasts (Davenport, H. E.) 471
Microsome fraction of guinea-pig liver, incorporation of radioactive amino acids into proteins of (Simkin, J. L. & Sutton, C. R.) 489
Microsomes, changes in components of, accompanying cell differentiation (Loening, U. E.) 20P
Microsomes in a system from rat liver *in vitro*, transfer of soluble ribonucleic acid to (Smith, C. J. & Reid, E.) 19P
Microsomes, ox-liver, presence of sulphatases A and B in (Roy, A. B.) 380
Mitochondria, distribution of glycolytic enzymes in preparations of (Johnson, M. K.) 610
Mitochondria, ox-liver, presence of sulphatases A and B in (Roy, A. B.) 380
Mitochondria, pea-root, polarographic measurement of oxygen uptake by (Stickland, R. G.) 636
(+)-Mollisacacidin, radial distribution of, in wattle heartwood (Roux, D. G. & Paulus, E.) 315
Mucopeptide biosynthesis by *Staphylococcus aureus* (Rogers, H. J. & Perkins, H. R.) 448
Muscle, amino acid-activating enzymes in (Pennington, R. J.) 205
Muscle, codling, acid-soluble nucleotides of (Jones, N. R. & Murray, J.) 567
Muscle, partial purification of carnosine–anserine synthetase of (Stenesh, J. J. & Winnick, T.) 575
Mustard gas, reaction of, with nucleic acids *in vitro* and *in vivo* (Brookes, P. & Lawley, P. D.) 478
Myofibrils, inhibitory action of relaxing-factor preparations on adenosine triphosphatase of (Baird, G. D. & Perry, S. V.) 262

2-[8-^{14}C]Naphthylamine, metabolic path of, in animals (Goldblatt, M. W., Henson, A. F. & Somerville, A. R.) 511
Neurospora crassa, further genetic variety of glutamic acid dehydrogenase in (Fincham, J. R. S. & Bond, P. A.) 96
Nicotinamide, metabolism of, by *Saccharomyces cerevisiae* (Sundaram, T. K., Rajagopalan, K. V., Pichappa, C. V. & Sarma, P. S.) 145
Nicotinic acid, metabolism of, by *Saccharomyces cerevisiae* and *Leuconostoc mesenteroides* (Sundaram, T. K., Rajagopalan, K. V., Pichappa, C. V. & Sarma, P. S.) 145
Nitrate reductase and related enzymes in *Escherichia coli* (Heredia, C. F. & Medina, A.) 24
Nitrate reduction in *Pseudomonas aeruginosa* (Fewson, C. A. & Nicholas, D. J. D.) 3P
Nitrite reductase from *Pseudomonas aeruginosa* (Walker, G. C. & Nicholas, D. J. D.) 4P
Nitrogen, photofixation of, by thiosulphate during bacterial photosynthesis (Arnon, D. I., Losada, M., Nozaki, M. & Tagawa, K.) 23P
C-Nitroso compounds, aromatic, reactions of, with haemoglobin (Gibson, Q. H.) 519
Nucleic acid in mammalian cells, inhibitor studies on time sequence of synthesis of (Paul, J. & Hagawira, A.) 21P
Nucleic acid, sites of synthesis of, in infected Krebs-2 ascites-tumour cells (Bellett, A. J. D. & Burness, A. T. H.) 17P
Nucleic acids, inhibition of incorporation of ^{14}C-labelled purines into, by a substituted pyrimidine (Newton, B. A.) 17P
Nucleic acids, reaction of mustard gas with, *in vitro* and *in vivo* (Brookes, P. & Lawley, P. D.) 478
Nuclei, enzymic composition of, isolated from radiosensitive and non-sensitive tissues with special reference to catalase activity (Creasey, W. A.) 5
Nucleotide sequences, frequencies of, in deoxyribonucleic acids (Burton, K.) 547
Nucleotides, acid-soluble, of codling muscle (Jones, N. R. & Murray, J.) 567
Oestradiol, changes in enzymes of uterus of ovariectomized rat after treatment with (Scott, D. B. M. & Lisi, A. G.) 52
Oestradiol-17β, quantitative determination of, in human urine (Givner, M. L., Bauld, W. S. & Vagi, K.) 406
Oestriol, quantitative determination of, in human urine (Givner, M. L., Bauld, W. S. & Vagi, K.) 406
Oestriol, quantitative fractionation of mixtures of, with other oestrogens (Givner, M. L., Bauld, W. S. & Vagi, K.) 400
Oestradiol-17β, quantitative fractionation of mixtures of, with other oestrogens (Givner, M. L., Bauld, W. S. & Vagi, K.) 400
16-*epi*Oestriol, quantitative determinations of, in human urine (Givner, M. L., Bauld, W. S. & Vagi, K.) 406
16-*epi*Oestriol, quantitative fractionation of mixtures of, with other oestrogens (Givner, M. L., Bauld, W. S. & Vagi, K.) 400
Oestrone, quantitative determination of, in human urine (Givner, M. L., Bauld, W. S. & Vagi, K.) 406
Oestrone, quantitative fractionation of mixtures of, with other oestrogens (Givner, M. L., Bauld, W. S. & Vagi, K.) 400
Organophosphorus poisoning, mode of recovery of brain cholinesterase *in vivo* after (Blaber, L. C. & Creasey, N. H.) 597
Organophosphorus poisoning, mode of recovery of erythrocyte cholinesterase *in vivo* after (Blaber, L. C. & Creasey, N. H.) 591
Osteoclastoma, phosphatase activity in (Jeffree, G. M.) 2P
Ovariectomized rat, changes in enzymes of uterus of, after treatment with oestradiol (Scott, D. B. M. & Lisi, A. G.) 52
16-Oxo-oestradiol-17β, quantitative fractionation of mixtures of, with other oestrogens (Givner, M. L., Bauld, W. S. & Vagi, K.) 400
Oxidase activity of caeruloplasmin, effects of ions and chelating agents on (Curzon, G.) 66
Oxygen uptake by pea-root mitochondria, polarographic measurement of (Stickland, R. G.) 636

Palitantin, biosynthesis of (Chaplen, P. & Thomas, R.) 91
Pea-root mitochondria, polarographic measurement of oxygen uptake by (Stickland, R. G.) 636
Permeability of large-granule membranes to mouse-liver catalase *in vitro* (Adams, D. H. & Burgess, E. A.) 247
Pernicious anaemia, reduction of oxidized glutathione in erythrocyte haemolysates in (Jocelyn, P. C.) 363
β-Phenylethanol, oxidation of, by alcohol dehydrogenase of liver (MacLeod, L. D.) 10P
Phosphatase activity in an osteoclastoma (Jeffree, G. M.) 2P
Phosphatase activity in bones of hyperparathyroid monkeys (Jeffree, G. M.) 2P
Phosphate esters, fractionation of, on ion-exchange resin by a system of pH-gradient elution (Wade, H. E.) 534
Phosphatidylserine, brain, infrared spectra of (Kimura, Y. & Nagai, Y.) 3
Phosphodiesterase, effect of, on capacity of soluble ribonucleic acid to accept amino acids (Herbert, E.) 14P
Phosphogluconate dehydrogenase, activity of, in rat uterus (Scott, D. B. M. & Lisi, A. G.) 52
Phospholipase A activity, estimation of, in aqueous systems (Magee, W. L. & Thompson, R. H. S.) 526
Phospholipids fatty acid composition of, from subcellular particles of rat liver (Macfarlane, M. G., Gray, G. M. & Wheeldon, L. W.) 625
Phospholipids of erythrocyte 'ghosts' (Dawson, R. M. C., Hemington, N. & Lindsay, D. B.) 226
Phospholipids of ox spleen with special reference to the fatty acid and fatty aldehyde compositions of the lecithin and kephalin fractions (Gray, G. M.) 82
Phosphoprotein phosphatase in cerebral tissues (Rose, S. P. R. & Heald, P. J.) 8P

Phosphorylating kinases of neoplastic and normal cells, some characteristics of (Bianchi, P., Butler, J. A. V., Crathorn, A. R. & Shooter, K. V.) 15P
Photosynthesis, bacterial, photofixation of nitrogen and photoproduction of hydrogen by thiosulphate during (Arnon, D. I., Losada, M., Nozaki, M. & Tagawa, K.) 23P
Plasma, human, insulin and an inhibitor of glucose uptake in protein fractions of (Vargas, L., Taylor, K. W. & Randle, P. J.) 43
Plasma, normal human-blood, nature of 'cholesterol' in (Greig, M. & Cook, R. P.) 16P
Polynucleotide phosphorylase, mode of action of (Grunberg-Manago, M.) 13P
Polynucleotide synthesis, chemical aspects of (Michelson, A. M.) 13P
Polypeptidyl gelatins, role of rigidity in antigenicity of (Sela, M. & Arnon, R.) 394
Polystyrene preparations, cross-linked, immunochemically active (Kent, L. H. & Slade, J. H. R.) 12
[4-^{14}C]Progesterone, 'conjugate' formation during metabolism of, by female rat liver *in vitro* (Cooke, B. A. & Taylor, W.) 21P
Progesterone metabolism in cat (Taylor, W. & Scratcherd, T.) 22P
Protease, purification and properties of a, from gut of *Etroplus suratensis* (Sundaram, S. & Sarma, P. S.) 465
Protein fractions of normal human plasma, insulin and an inhibitor of glucose uptake in (Vargas, L., Taylor, K. W. & Randle, P. J.) 43
Protein in infected Krebs-2 ascites-tumour cells, sites of synthesis of (Bellett, A. J. D. & Burness, A. T. H.) 17P
Protein intake, effect of, on ribonucleic acid of liver-cell sap (Prosser, E. J. T., Mallinson, A. C. & Munro, H. N.) 24P
Protein of rat diaphragm, influence of alloxan-diabetes on incorporation of amino acids into (Manchester, K. L. & Young, F. G.) 386
Protein solutions, effect of, on oxidation of glutathione in oxygenated erythrocytes (Jocelyn, P. C.) 368
Protein synthesis in mammalian cells, inhibitor studies on time sequence of (Paul, J. & Hagiwara, A.) 21P
Protein, synthesis of, in a purine-requiring *Escherichia coli* infected with bacteriophage T2 (Fessler, L. I., Kelemen, M. V. & Burton, K.) 558
Proteins, attachment of, to cells tanned with pyruvic aldehyde (Ling, N. R.) 12P
Proteins, incorporation of radioactive amino acids into, of microsome fraction of guinea-pig liver (Simkin, J. L. & Sutton, C. R.) 489
Pseudomonas aeruginosa, degradation and synthesis of γ-aminobutyric acid by (Bachrach, U.) 417
Pseudomonas aeruginosa, nitrate reduction in (Fewson, C. A. & Nicholas, D. J. D.) 3P
Pseudomonas aeruginosa, nitrite reductase from (Walker, G. C. & Nicholas, D. J. D.) 4P
Pseudomonas cytochrome *c*-551, crystalline, preparation of, and its general properties (Horio, T., Higashi, T., Sasagawa, M., Kusai, K., Nakai, M. & Okunuki, K.) 194
Purines, ^{14}C-labelled, inhibition of incorporation of, into nucleic acids by a substituted pyrimidine (Newton, B. A.) 17P
β-Pyrazol-1-ylalanine from water-melon seeds (Noe, F. F. & Fowden, L.) 543
β-Pyrazol-1-ylalanine, nuclear-magnetic-resonance spectrum of (Ridd, J. H. & White, R. F. M.) 546
Pyridine nucleotide, studies on biosynthesis of (Sundaram, T. K., Rajagopalan, K. V., Pichappa, C. V. & Sarma, P. S.) 145
Pyrimidine metabolism in parasitic flatworms (Campbell, J. W.) 105
Pyruvic aldehyde, attachment of proteins to cells tanned with (Ling, N. R.) 12P
Relaxing-factor preparations, inhibitory action of, on myofibrillar adenosine triphosphatase (Baird, G. D. & Perry, S. V.) 262
Ribonucleic acid, biosynthesis of (Burdon, R. H.) 14P
Ribonucleic acid of liver-cell sap, effect of protein intake on (Prosser, E. J. T., Mallinson, A. C. & Munro, H. N.) 24P
Ribonucleic acid, soluble, effect of phosphodiesterase on capacity of, to accept amino acids (Herbert, E.) 14P
Ribonucleic acid, soluble, pyrophosphorolysis of (Canellakis, E. S.) 14P
Ribonucleic acid, soluble, transfer of, to microsomes in a system *in vitro* from rat liver (Smith, C. J. & Reid, E.) 19P
Ribonucleotides, acid-soluble, levels and distribution of, in mammary glands of pregnant and lactating rats (Wang, D. Y. & Greenbaum, A. L.) 552
Ring D α-ketolic oestrogens, quantitative determination of, in human urine (Givner, M. L., Bauld, W. S. & Vagi, K.) 406
Ring D α-ketolic oestrogens, quantitative fractionation of mixtures of, with other oestrogens (Givner, M. L., Bauld, W. S. & Vagi, K.) 400

Saccharomyces cerevisiae, metabolism of nicotinic acid and nicotinamide by (Sundaram, T. K., Rajagopalan, K. V., Pichappa, C. V. & Sarma, P. S.) 145
Saccharomyces cerevisiae, purification and properties of tyrosine–2-oxoglutarate transaminase of (SentheShanmuganathan, S.) 619
Salicin, formation of *o*-hydroxybenzyl β-glucopyranoside from (Anderson, J. D., Hough, L. & Pridham, J. B.) 564
Salicylate congeners and uncoupling activity (Brostoff, D. V., Moses, V. & Smith, M. J. H.) 5P
Sedimentation coefficients, a graphical method for rapid determination of (Markham, R.) 516
L-[3-^{14}C]Serine, preparation of (Batt, R. D., Dickens, F. & Williamson, D. H.) 272
Serine residue, reactive, amino acid sequence around, of proteolytic enzymes (Naughton, M. A., Sanger, F., Hartley, B. S. & Shaw, D. C.) 149
Serine transhydroxymethylase, spectrophotometric study of reaction catalysed by (Blakley, R. L.) 459
Silica, soluble, micro-determination of, in urine (Paul, J.) 202
Skin, effect of growth on chemical composition of (Widdowson, E. M. & Dickerson, J. W. T.) 30
Skin, mechanism of glucuronide synthesis in (Stevenson, I. H. & Dutton, G. J.) 19P
Spinach chloroplasts, synthesis of glycollic acid by (Zelitch, I.) 11P
Spleen, ox, phospholipids of, with special reference to the fatty acid and fatty aldehyde compositions of the lecithin and kephalin fractions (Gray, G. M.) 82
Staphylococcus aureus, 5-fluorouracil and mucopeptide biosynthesis by (Rogers, H. J. & Perkins, H. R.) 448
Sulphatases A and B, intracellular distribution of (Roy, A. B.) 380
Sulphate acceptors, endogenous, in rat liver (Spencer, B.) 294
Sulphite, effect of, on polymerization of urease (Creeth, J. M. & Nichol, L. W.) 230
Sulphite in fungi (Spencer, B. & Harada, T.) 305
Sulphokinase in rat liver (Spencer, B.) 294
Sulphur metabolism of fungi, role of choline sulphate in (Spencer, B. & Harada, T.) 305

Taurocyamine of mammalian brain (Blass, J. P.) 484
Tetrahydropteroylglutamic acid, function of, in the reaction catalysed by serine transhydroxymethylase (Blakley, R. L.) 459
[^{14}C]Tetritols, preparation and degradation of specifically labelled (Batt, R. D., Dickens, F. & Williamson, D. H.) 272

Tetritols, utilization of, by rat tissues (Batt, R. D., Dickens, F. & Williamson, D. H.) 281
[^{14}C]Tetroses, preparation and degradation of specifically labelled (Batt, R. D., Dickens, F. & Williamson, D. H.) 272
Tetroses, utilization of, by rat tissues (Batt, R. D., Dickens, F. & Williamson, D. H.) 281
Thiosulphate, photofixation of nitrogen and photoproduction of hydrogen by, during bacterial photosynthesis (Arnon, D. I., Losada, M., Nozaki, M. & Tagawa, K.) 23P
L-Threitol, preparation of specifically labelled (Batt, R. D., Dickens, F. & Williamson, D. H.) 272
Thymine, degradation of, by rat tapeworm (Campbell, J. W.) 105
Thymus, calf, improved fractionations of arginine-rich histones from (Johns, E. W., Phillips, D. M. P., Simpson, P. & Butler, J. A. V.) 631
Thyroxine dehalogenase, partial purification and properties of (Tata, J. R.) 214
Tobacco mosaic virus, reaction *in vitro* and *in vivo* of nucleic acids of, with mustard gas (Brookes, P. & Lawley, P. D.) 478
Torula, effects of prolonged oxygenation on the cytochrome and enzyme systems of (Scutt, P. B.) 6P
Transhydrogenase, activity of, in rat uterus (Scott, D. B. M. & Lisi, A. G.) 52
1:1:1-Trichloro-2:2-di-(*p*-chlorophenyl)ethane (DDT) and rat adipose tissue (Parker, V. H.) 74
Triphosphopyridine nucleotide, protein from leaves catalysing reduction of, by illuminated chloroplasts (Davenport, H. E.) 471
Trypsin, di*iso*propoxy[^{32}P]phosphinyl derivatives of (Naughton, M. A., Sanger, F., Hartley, B. S. & Shaw, D. C.) 149
Tumour cells, infected Krebs-2 ascites, sites of synthesis of a nucleic acid and a protein in (Bellett, A. J. D. & Burness, A. T. H.) 17P
Tumour, Ehrlich ascites, reaction *in vitro* and *in vivo* of nucleic acids of, with mustard gas (Brookes, P. & Lawley, P. D.) 478
Tungsten, determination of, in biological material by activation analysis (Bowen, H. J. M.) 79
Tyrosine in infants on a normal diet, an inborn defect in metabolism of (Bloxam, H. R., Day, M. G., Gibbs, N. K. & Woolf, L. I.) 320
Tyrosine–2-oxoglutarate transaminase, of *Saccharomyces cerevisiae*, purification and properties of (SentheShanmuganathan, S.) 619
Tyrosine *O*-sulphate in the rat, metabolism of (Dodgson, K. S., Powell, G. M., Rose, F. A. & Tudball, N.) 20P

Uracil, degradation of, by rat tapeworm (Campbell, J. W.) 105
Urease, chemical interaction of, in solution (Creeth, J. M. & Nichol, L. W.) 230
Urine colloids, fractionation of, on anion-exchange cellulose (Anderson, A. J., Lepper, M. H. & Winzler, R. J.) 581
Urine, human, quantitative determination of 2-methoxyoestrone, oestrone, ring D α-ketolic oestrogens, oestradiol-17β, 16-*epi*oestriol and oestriol in (Givner, M. L., Bauld, W. S. & Vagi, K.) 406
Urine, micro-determination of soluble silica in (Paul, J.) 202
Uterus of ovariectomized rat, changes in enzymes of, after treatment with oestradiol (Scott, D. B. M. & Lisi, A. G.) 52

Vitamin E-deficient erythrocytes, lipid peroxidation in dialuric acid-induced haemolysis of (Bunyan, J., Green, J., Edwin, E. E. & Diplock, A. T.) 47

Water-melon seeds, β-pyrazol-1-ylalanine from (Noe, F. F. & Fowden, L.) 543

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **396th Meeting** *of the Society was held in the Department of Physiology, University of Bristol, on Friday,* 15 *July* 1960, *when the following papers were read:*

COMMUNICATIONS

Inhibition and Action Pattern of β-Amylase in Presence of Maltose

By U. K. Misra and Dexter French (introduced by W. J. Whelan). (*Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa, U.S.A.*)

Kuhn (1925) reported the inhibition of malt and pancreatic amylases by maltose. In the present report we have studied the typical inhibition and the change in action pattern of β-amylase in presence of varying concentrations of maltose. A commercial sweet-potato β-amylase preparation was used as a source of enzyme and soluble potato starch as a substrate. The degree of β-amylolysis of potato starch in presence and absence of varying amounts of added maltose was followed by iodine staining of the hydrolysis products and measuring their absorption spectra in a Cary spectrophotometer (Model 14). The reaction mixture, pH 4·8, was incubated at 35° and samples of digest (1 ml. each) were taken out at regular intervals, inactivated, stained with iodine and their absorption spectra measured. The action pattern of β-amylase was studied by the slope analysis (Bailey & Whelan, 1957) of the curves and the degree of inhibition by the rate of change in optical density of the iodine-stained products. An inference of the action pattern of β-amylase can be derived from the slope of the line passing through these maxima of the spectral curves, which, if zero, indicates a single-chain attack and if greater than zero, a multi-chain attack. The kinetic results indicated that maltose is a competitive inhibitor of β-amylase (K_m = 0·27% and K_i = 0·006 M). As regards the change in action patterns of β-amylase in presence of varying amounts of added maltose, the results do not furnish conclusive evidence in favour of or against one or the other type of action patterns. But the results imply that in an uninhibited system, or one containing a low inhibitor concentration, β-amylase operates in a multi-chain fashion. As the concentration of inhibitor is raised, the action pattern appears to remain essentially the same, or if it shifts at all it is toward a single-chain pattern.

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REFERENCES

Bailey, J. M. & Whelan, W. J. (1957). *Biochem. J.* **67**, 540.
Kuhn, R. (1925). *Liebigs Ann.* **1**, 443.

The Response to Increased Magnesium Intake

By F. W. Heaton. (*Medical Research Council Unit for Metabolic Disturbances in Surgery, The General Infirmary, Leeds*)

Although the activating effect of magnesium on many enzyme systems is well established (Wacker & Vallee, 1958), much less is known about its relationship to other body constituents. Evidence has been presented for a connexion between the serum concentrations of magnesium and calcium (Brookfield, 1934) or potassium (Smith, 1951), while other workers have reported variable effects on the urinary excretion of other cations after infusing magnesium salts (Chesley & Tepper, 1958; Womersley, 1956, 1958).

Balance techniques have been used to investigate the effects of administering oral supplements of up to 500 mg. of magnesium daily to normal subjects maintained on an otherwise constant diet of normal composition. The amount of magnesium absorbed was directly proportional to the amount ingested within the threefold range of intake studied and positive balance occurred during the periods of high intake. The maximum rise in urinary magnesium excretion was delayed for approximately 48 hr. after the increase in intake and remained elevated for 48 hr. after cessation of the supplement. No change in the serum concentration of magnesium or any other cation was observed.

Increased magnesium intake was accompanied by elevated urinary excretion of calcium as previously observed by Tibbetts & Aub (1937), but there was also reduced faecal excretion of calcium, the net result being positive calcium balance. The increase in urinary calcium preceded the rise in magnesium excretion, reaching a maximum after supplementation for 24 hr., and then partially declining to a still elevated level. A further increase in magnesium intake at this point caused an additional rise in calcium excretion.

In addition to the effect on calcium metabolism, raised magnesium intake also stimulated positive

balance of both potassium and nitrogen. The effect on nitrogen balance was due mainly to reduced urinary excretion of nitrogen, which persisted well into the recovery phase after cessation of the supplement. No significant variation in urinary potassium excretion was observed, the positive balance being due to reduced faecal loss which was dependent on the continuation of high magnesium intake. The changes in magnesium and potassium balance were greater than required by the nitrogen balance for muscle formation (Dickerson & Widdowson, 1960). No effect on sodium metabolism was observed.

REFERENCES

Brookfield, R. W. (1934). *Biochem. J.* **28**, 725.
Chesley, L. C. & Tepper, I. (1958). *J. clin. Invest.* **37**, 1362.
Dickerson, J. W. T. & Widdowson, E. M. (1960). *Biochem. J.* **74**, 247.
Smith, S. G. (1951). *Amer. J. Physiol.* **164**, 702.
Tibbetts, D. M. & Aub, J. C. (1937). *J. clin. Invest.* **16**, 491.
Wacker, W. E. C. & Vallee, B. L. (1958). *New Engl. J. Med.* **259**, 431, 475.
Womersley, R. A. (1956). *Clin. Sci.* **15**, 465.
Womersley, R. A. (1958). *J. Physiol.* **143**, 300.

Phosphatase Activity in Bones of Hyperparathyroid Monkeys

By Grace M. Jeffree. (*University of Bristol Pathology Research Laboratory, 18 Guinea Street, Bristol, 1*)

Limb bones from three specimens of Humbolt's Woolly Monkey were examined for phosphatase activity. All three animals appeared to have suffered from hyperparathyroidism of different degrees of severity. Brief findings on the three individuals follow.

A. Adolescent female; epiphyses still open; body weight 2·698 kg. Bones appear normal, firm, well-calcified and free from fractures, but serum chemistry 3 months before death suggested hyperparathyroidism: Ca, 12·1 mg./100 ml.; P, 9·4 mg./100 ml.; alkaline phosphatase 296 King-Armstrong units/100 ml. Wet wt. of parathyroid tissue at death, 29 mg. (10·7 mg./kg. of body wt.). Acid and alkaline phosphatase activities in femur roughly comparable to those of a 6-month-old rabbit.

B. Adolescent male, epiphyses still open; body weight 4·536 kg. No blood chemistry. Wet wt. of parathyroid tissue, 44 mg. (9·7 mg./kg. of body wt.). Bones markedly softened, with some pathological fractures. Alkaline phosphatase levels in femur greatly raised, to concentrations greater than those in the femur of a 3-week-old rabbit. Acid phosphatase levels roughly comparable with those of *A*.

C. Adolescent male, epiphyses still open; body weight 1·927 kg. No blood chemistry. Wet wt. of parathyroid tissue, 91 mg. (47 mg./kg. of body wt.). Bone changes very far advanced; many pathological fractures, much deformity; bones very soft. Alkaline phosphatase levels in femur between those of *A* and *B*; acid phosphatase levels somewhat lower than *A* or *B*. Minimal activity of both alkaline and acid phosphatase in fractured head of femur.

Alkaline phosphatase tends to be concentrated below the periosteum, in the lower layers of articular and epiphyseal cartilage and in the intertrabecular cells of cancellous bone. The pattern of acid phosphatase distribution is somewhat similar at markedly lower levels of activity. Osteoclasts are not frequent in any of the bones examined.

Phosphatase Activity in an Osteoclastoma

By Grace M. Jeffree. (*University of Bristol Pathology Research Laboratory, 18 Guinea Street, Bristol, 1*)

Demonstration of phosphatase in a malignant osteoclastoma by the methods of Burstone (1958*a*, *b*) shows high acid phosphatase activity in osteoclasts and some in the stroma. It is suspected that some part of this acid phosphatase activity in the stroma is due to diffusion of the enzyme from the osteoclasts. Alkaline phosphatase activity is absent from the osteoclasts, but is very high in the stroma.

Quantitative estimations of phosphatase using *p*-nitrophenyl phosphate as substrate show enzyme activity at pH 10·3 to be of the order of 20 times the activity at pH 4·9. Alkaline phosphatase activity is somewhat higher in material that is mainly composed of necrotic tissue and blood clot than in viable tumour tissue, whereas acid phosphatase activity tends to be slightly higher in viable tumour tissue than in necrotic material. *p*-Nitrophenyl phosphate was hydrolysed at rates up to 3875 μmoles/hr./g. of tissue at 37° and pH 10·3; and up to 132 μmoles/hr./g. of tissue at pH 4·9. These rates compare with those of regions of most active bone formation in the femur of a 3-week-old rabbit. Notwithstanding the very high alkaline phosphatase activity, the tumour was osteolytic in character.

The work reported in these two communications was supported by a grant from the British Empire Cancer Campaign. The author thanks Dr C. H. G. Price for his very great help in interpreting histological findings, and Mr J. E. Hancock for the preparation of slides.

REFERENCES

Burstone, M. S. (1958*a*). *J. nat. Cancer Inst.* **20**, 601.
Burstone, M. S. (1958*b*). *J. nat. Cancer Inst.* **21**, 523.

The Absence of Galactosamine in Scorbutic Granulation Tissue

By R. E. HUGHES* and E. KODICEK. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

Granulation tissue (Kodicek & Loewi, 1955) and cartilage (Reddi & Norström, 1954) of scorbutic guinea pigs show, apart from the impaired formation of collagen, a reduced incorporation of $^{35}SO_4^{2-}$ into chondroitin sulphates. This metabolic defect was considered to be caused by a block in their biosynthesis (Kodicek & Loewi, 1955). On the other hand, Friberg (1958) concluded from paired-feeding experiments that the decreased sulphate uptake was caused mainly by inanition.

The present experiments were undertaken to ascertain whether any change occurred in the ratio of galactosamine to glucosamine in granulation tissue during scurvy. The tissue formed after tendonectomy, as well as costal cartilage, was obtained from 120 normal, paired-fed and scorbutic guinea pigs. The dried material, was hydrolysed in 4N-HCl for 15 hr. at 100°, fractionated by chromatography on Dowex 50 (Boas, 1953), and analysed for its galactosamine and glucosamine content by the triphenyltetrazolium method (Fischer & Dörfel, 1954) on paper chromatograms run in ethyl acetate–water–pyridine (2:2:1, by vol.). The results agreed well with those of samples digested with papain and trypsin, and precipitated with 75–96% ethanol. Good agreement was also obtained with the estimation of total hexosamines by the method of Elson & Morgan (1933).

Scorbutic granulation tissue had little or no galactosamine, while that from paired-fed or normal animals had 0·27%. No significant differences were observed between scorbutic, paired-fed and normal animals in the glucosamine content (0·36, 0·50, 0·61% respectively). The granulation tissue of paired-fed animals resembled that of normal controls in its histological appearance and in the amounts formed (67·0 and 60·4 mg. dry wt./animal, respectively), while in scurvy only 18·3 mg. were produced and it had the abnormal histological features previously described (Bradfield & Kodicek, 1951).

In scorbutic and normal costal cartilage, no significant difference was found in the galactosamine (3·17 and 3·36%) or glucosamine content (0·51 and 0·41%).

The absence of galactosamine in scorbutic granulation tissue and its presence in that of paired-fed controls indicates an impaired formation, not associated with inanition, of chondroitin-containing mucopolysaccharides. In cartilage, this defect might be masked by the large amount of preformed chondroitin-containing mucopolysaccharide that has a slower turnover rate than in granulation tissue (Junge-Hülsing, 1959). On the other hand, the uptake of $^{35}SO_4^{2-}$, being connected with newly forming chondroitin-containing mucopolysaccharides, might be more severely affected.

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REFERENCES

Boas, N. F. (1953). *J. biol. Chem.* **204**, 553.
Bradfield, J. R. G. & Kodicek, E. (1951). *Biochem. J.* **49**, xvii.
Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
Fischer, F. G. & Dörfel, H. (1954). *Biochem. Z.* **297**, 164.
Friberg, U. (1958). *Arkiv. Kemi*, **12**, 501.
Junge-Hülsing, G. (1959). *Z. Rheumaforsch.* **18**, 355.
Kodicek, E. & Loewi, G. (1955). *Proc. Roy. Soc.* B, **144**, 100.
Reddi, K. K. & Norström, A. (1954). *Nature, Lond.*, **173**, 1232.

Nitrate Reduction in *Pseudomonas aeruginosa*

By C. A. FEWSON and D. J. D. NICHOLAS* (*Long Ashton Research Station, University of Bristol*)

Nitrate reductases from *Neurospora*, *Escherichia coli* and green plants have been shown to be molybdo-flavoproteins (Nicholas & Nason, 1954, 1955*a*, *b*). In addition, cytochromes have been implicated in nitrate reduction in *E. coli* and in some dentrifying bacteria (Taniguchi *et al.* 1957; Verhoeven & Takeda, 1956). In this communication we show that both cytochrome and molybdenum are required for nitrate reduction in actively denitrifying *Pseudomonas aeruginosa* (NCIB 8704).

Cells grown on a peptone–nitrate medium (Verhoeven, 1952) under anaerobic conditions were disrupted by the ultrasonic probe (20 kcycles/sec.) and most of the nitrate reductase was located in the supernatant solution after centrifuging at 20000 *g* for 45 min. The enzyme was purified 115-fold by ammonium sulphate precipitation, protamine sulphate and calcium phosphate gel treatments, and electrophoresis on granular starch. The purified enzyme reduced nitrate (K_m NO_3^- = $1{\cdot}6 \times 10^{-5}$M) with reduced diphosphopyridine nucleotide (DPNH) (K_m DPNH = $4{\cdot}2 \times 10^{-5}$M), but not with reduced triphosphopyridine nucleotide, as a hydrogen donor.

Cells deficient in either molybdenum or iron showed a marked decrease in nitrate reductase activity, whereas deficiencies of other metals were

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without effect. When ^{99}Mo or ^{59}Fe were incorporated into the culture medium both metals concentrated in the purified enzyme to the extent of 5 μg. of Fe and 0·15 μg. of Mo/mg. protein.

A flavin requirement for the enzyme was shown after its repeated precipitation with ammonium sulphate (pH 5·5). The addition of flavinadenine dinucleotide (FAD) (K_m FAD $= 2 \times 10^{-7}$ M) to this apo-enzyme restored its activity to the original level; flavine mononucleotide was about 50% as effective as FAD. Sulphydryl groups appear to be involved in binding the flavin to the protein moiety since *p*-chloromercuribenzoate competitively inhibited stimulation of the apo-enzyme by FAD.

The purified enzyme contained cytochrome *c* which was reduced by DPNH; Mo^{6+} or NO_3^- re-oxidized the cytochrome. A requirement for mammalian or bacterial cytochrome *c* was obtained by repeated precipitation of the purified enzyme with ammonium sulphate (pH 8·2). Reduced mammalian cytochrome *c* was an effective donor for the reduction of nitrate. A DPNH-dependent cytochrome *c* reductase paralleled nitrate reductase activity during enzyme purification, heat denaturation and growth at various oxygen tensions. The activation energies of both enzyme systems were identical, i.e. 6500 cal./mole above 19° and 12000 cal./mole below 19°.

Nitrate reductase activity in cell homogenates was decreased with increased oxygen tension but this effect was offset by pretreating the extract with carbon monoxide, presumably because the latter inhibited cytochrome oxidase. Similar results have been reported by Stickland (1931) in *E. coli*.

A possible sequence of electron transfer during nitrate reductase action in *P. aeruginosa* is:

$$\text{DPNH} \rightarrow \text{FAD} \rightarrow \text{cytochrome } c \rightarrow \text{Mo} \rightarrow NO_3^-$$
$$\downarrow$$
$$\text{cytochrome oxidase}$$
$$\downarrow$$
$$O_2$$

REFERENCES

Nicholas, D. J. D. & Nason, A. (1954). *J. biol. Chem.* **211**, 183.

Nicholas, D. J. D. & Nason, A. (1955*a*). *Plant Physiol.* **30**, 135.

Nicholas, D. J. D. & Nason, A. (1955*b*). *J. Bact.* **69**, 580.

Stickland, L. H. (1931). *Biochem. J.* **25**, 1543.

Taniguchi, S., Asano, A., Iida, K., Kono, M., Ohmachi, K. & Egami, F. (1957). *Intern. Symp. Enzyme Chem., Tokyo*, **2**, 238.

Verhoeven, W. (1952). Thesis, University of Delft.

Verhoeven, W. & Takeda, Y. (1956). In *Inorganic Nitrogen Metabolism*, p. 159. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.

Nitrite Reductase from *Pseudomonas aeruginosa*

By G. C. Walker and D. J. D. Nicholas.* (*Long Ashton Research Station, University of Bristol*)

It has been shown that nitrite reductase from bacteria, fungi or higher plants is a metallo-flavoprotein (Yamagata, 1940; Taniguchi, Mitsui, Toyoda & Egami, 1952; Nason, Abraham & Averbach, 1954; Spencer, Takahashi & Nason, 1957; Medina & Nicholas, 1957; Asano, 1959). In *Pseudomonas stutzeri* (Chung & Najjar, 1956) and in *Neurospora crassa* (Nicholas, Medina & Jones, 1960) the enzyme requires Fe and Cu for its activity.

We now show that crude extracts of *P. aeruginosa* (NCIB 8704), grown in still culture (Verhoeven, 1952), rapidly reduced nitrite anaerobically in the presence of either reduced diphosphopyridine nucleotide (DPNH), reduced flavinadenine dinucleotide ($FADH_2$), reduced flavine mononucleotide ($FMNH_2$), reduced pyocyanine (PyH_2) or reduced methylene blue (MbH_2). The product of the reaction was nitrogen.

A 600-fold purification of the enzyme was obtained by ammonium sulphate precipitation and calcium phosphate gel treatment. A strong inhibitor present in the crude extract reduced enzyme activity to 10% of that in a 0–100% saturated ammonium sulphate precipitate. Deficiency of Cu or Fe depressed enzyme activity whereas a deficiency of Mn or Mo was without effect. Either ^{59}Fe or ^{64}Cu, when incorporated into the culture medium, concentrated in the purified enzyme (40 μm-moles of Fe and 1·0 μm-moles of Cu/mg. of protein). The purified enzyme was yellow-green ($\lambda_{max.}$ 635 mμ) with a cytochrome *c*-type spectrum ($\lambda_{max.}$ 410, 520, 550 mμ). The peak at 635 mμ was diminished by dialysis against mM-potassium cyanide and restored by adding 2 μm-moles of Cu/mg. of protein but Co, Ni, Zn, Mn and Fe were without effect. The following were effective electron donors for the purified enzyme: $FADH_2$, $FMNH_2$, PyH_2 and MbH_2, but DPNH and TPNH were without effect (K_m $FADH_2 \equiv FMNH_2 \equiv PyH_2 \equiv 8 \times 10^{-8}$ M; K_m $MbH_2 \equiv 3 \times 10^{-7}$ M). The 635 mμ peak substance, after reduction by the electron donors, was oxidized rapidly by NO_2^-, slowly by O_2, and not at all by NO_3^- and NH_2OH. Cytochrome in the enzyme was not reduced. FAD in the purified enzyme (1·5 μm-moles/mg. of protein) could be partially removed by treatment with ultraviolet light (6 hr.) and precipitation with ammonium sulphate (pH 6·0). When PyH_2 was the electron donor, FAD or FMN (10^{-7} M) increased activity 100 and 30% respectively, but there was no effect when MbH_2 was used. A PO_4^{3-} requirement for maximal activity

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was replaced by SO_4^{2-} but not by AsO_4^{3-}, $P_2O_7^{4-}$, MoO_4^{-}, BO_3^{3-}, WO_4^{2-}, or SiO_4^{4-}. Inhibition of the enzyme by *p*-chloromercuribenzoate was reversed by glutathione or cysteine indicating the presence of sulphydryl groups.

The product of NO_2^- reduction (K_m $NO_2^- \equiv 3{\cdot}1 \times 10^{-5}$ M) was NO, since 1 μmole of NO_2^- produced 22·4 μl. of gas which was absorbed completely by alkaline Na_2SO_3. The stoicheiometry of the reaction is

$$FADH_2 + 2NaNO_2 \rightarrow 2NO + FAD + 2NaOH.$$

REFERENCES

Asano, A. (1959). *J. Biochem., Tokyo*, **46**, 781.
Chung, C. W. & Najjar, V. A. (1956). *J. biol. Chem.* **218**, 617.
Medina, A. & Nicholas, D. J. D. (1957). *Biochim. biophys. Acta*, **25**, 138.
Nason, A., Abraham, R. G. & Averbach, B. C. (1954). *Biochim. biophys. Acta*, **15**, 159.
Nicholas, D. J. D., Medina, A. & Jones, O. T. G. (1960). *Biochim. biophys. Acta*, **37**, 468.
Spencer, D., Takahashi, H. & Nason, A. (1957). *J. Bact.* **73**, 553.
Taniguchi, S., Mitsui, T., Toyoda, T. & Egami, F. (1952). *J. Biochem., Tokyo*, **40**, 175.
Verhoeven, W. (1952). Thesis, University of Delft.
Yamagata, S. (1940). *Acta Phytochim., Tokyo*, **11**, 145.

Salicylate Congeners and Uncoupling Activity

By D. V. Brostoff,* V. Moses† and M. J. H. Smith. (*Department of Chemical Pathology, King's College Hospital Medical School, London, S.E.* 5)

Salicylate has been shown to uncouple oxidative phosphorylation reactions in respiring mitochondrial preparations (Brody, 1956) and to produce effects on the metabolism of isolated tissues and yeast which are directly explicable in terms of this uncoupling action (Smith & Moses, 1960; Moses & Smith, 1960). However, the relation between chemical structure and uncoupling activity in the salicylate group of compounds has not been studied in any detail. 2:3-Dihydroxybenzoic acid, thiosalicylic acid and methyl salicylate have been reported to inhibit phosphorylation but not oxidation in mitochondrial preparations (Brody, 1956; Packer, Austen & Knoblock, 1959) and the cresotic acids have been observed to cause increased oxygen consumption in the intact rat (Andrews, 1958).

We have used a suspension of starved baker's yeast cells incubated with a known amount of glucose as a test system for uncoupling activity. The oxygen consumption of the suspension accounts for only about 30% of the amount theoretically required for the complete oxidation of all the glucose to CO_2, the remainder of the glucose presumably being assimilated (Pickett & Clifton, 1943). Uncoupling reagents, such as 2:4-dinitrophenol, decrease the proportion of the glucose which is assimilated and increase the proportion oxidized, hence stimulating the oxygen consumption of the preparation (Clifton, 1946).

A number of salicylate congeners were tested over a concentration range varying from 0·1 mM to 20 mM. Dihydroxybenzoic acids and hexahydrosalicylic acid had no effect at any of the concentrations tested whereas *o*-methoxybenzoic and 3-phenylsalicylic acids caused a marked inhibition of the oxygen consumption at the higher concentrations. 2:4-Dinitrophenol and salicylate caused a stimulation followed by an inhibition of the oxygen consumption with increasing concentration. This response was also produced by salicylaldehyde, *m*-cresotic acid and 1-hydroxy-2-naphthoic acid. Some implications of these findings were discussed.

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REFERENCES

Andrews, M. M. (1958). *Brit. J. Pharmacol.* **13**, 419.
Brody, T. M. (1956). *J. Pharmacol.* **117**, 39.
Clifton, C. E. (1946). *Advanc. Enzymol.* **6**, 269.
Moses, V. & Smith, M. J. H. (1960). *Biochem. J.* **74**, 18P.
Packer, L., Austen, F. K. & Knoblock, E. C. (1959). *Proc. Soc. exp. Biol., N.Y.*, **100**, 239.
Pickett, M. J. & Clifton, C. E. (1943). *J. cell. comp. Physiol.* **21**, 77.
Smith, M. J. H. & Moses, V. (1960). *Biochem. J.* **74**, 17P.

Anti-Inflammatory Drugs and Intermediary Metabolism

By V. Moses* and M. J. H. Smith. (*Department of Chemical Pathology, King's College Hospital Medical School, London, S.E.* 5)

Many of the effects of salicylate on the incorporation of ^{14}C from labelled substrates into the soluble metabolic intermediates of chopped preparations of isolated rat tissues are explicable in terms of an uncoupling action on oxidative phosphorylation reactions (Smith & Moses, 1960*a*, *b*). However, the anti-inflammatory properties of salicylate must be mediated by mechanisms other than an uncoupling action since 2:4-dinitrophenol (DNP), although a more powerful uncoupling reagent than salicylate, lacks anti-inflammatory effects (Adams & Cobb, 1958). Huggins, Moses & Smith (1960) found a difference between the effects of salicylate and DNP on the distribution of ^{14}C in the metabolic intermediates of rat-tissue preparations incubated

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with [3-^{14}C]pyruvate. Salicylate, but not DNP, caused a relative accumulation of radiocarbon in glutamic acid and a decreased incorporation into alanine. We have studied the effects of other anti-inflammatory drugs (butazolidin, chloroquine, hydrocortisone, and dexamethasone) on the metabolic patterns of isolated tissues to determine if they share a common action on tissue metabolism.

None of the substances (used at a concentration of 0·5 μg./ml. for dexamethasone, and 10 μg./ml. for the others) had any effect on the distribution of the incorporated ^{14}C from [^{14}C]glucose and [2-^{14}C]-acetate in brain preparations or from [3-^{14}C]pyruvate or [2-^{14}C]acetate in liver preparations. Using [^{14}C]glucose and liver, all the drugs caused a marked fall in the incorporation of radiocarbon in alanine and an increased appearance of the isotope into maltose, lactate, glycerate, cystine, aspartate and glutamate. Thus, although the anti-inflammatory drugs investigated in the present work had similar actions on intermediary metabolism there were few common features between them and salicylate. Unless the inhibition of alanine formation from [^{14}C]-glucose, which was reduced by some 85% in the present investigation, and 24% by salicylate (Smith & Moses, 1960*b*), should prove significant in further studies, an explanation of the anti-inflammatory actions possessed by all these drugs must probably be sought elsewhere.

REFERENCES

Adams, S. S. & Cobb, R. (1958). *Nature, Lond.*, **181**, 773.
Huggins, A. K., Moses, V. & Smith, M. J. H. (1960). *Biochem. J.* (in the Press).
Smith, M. J. H. & Moses, V. (1960*a*). *Biochem. J.* **74**, 17P.
Smith, M. J. H. & Moses, V. (1960*b*). *Biochem. J.* (in the Press).

The Periodate Oxidation of Amino Sugars and Related Compounds

By M. Cantley and L. Hough. (*Department of Organic Chemistry, The University, Bristol*)

Consideration of the anomalous oxidations reported for amino sugars and their *N*-acetyl derivatives suggested that ammonium and acetamido-malonaldehyde intermediates participate in these reactions. When 2-acetamido-2-deoxyaldoses (about 2 mM) were oxidized with sodium metaperiodate (15 mM) at room temperature and at pH values of 1–7·5, the major pathway was found to proceed via acetamido-malonaldehyde, giving, as in the case of *O*-substituted malonaldehydes (Cantley, Hough & Pittet, 1959), 0·7–0·9 mole of CO_2 with the consumption of 5·5–6·0 moles of periodate.

Interpretation of similar oxidations of 2-amino-2-deoxy sugars is complicated by equilibration of the free base with its ammonium ion, the proportions varying with pH. An unprotonated 2-amino alcohol is cleaved by periodate whereas the protonated species is resistant to oxidation (cf. McCasland & Smith, 1951) as shown by the decrease in the second-order rate constant for the oxidation of ethanolamine from 260 l. mole^{-1}.min.$^{-1}$ at pH 7·5 to 0·0166 l. mole^{-1}.min.$^{-1}$ at pH 1·2. (The initial concentrations of free ethanolamine at pH 7·5 and pH 1·2 are calculated to be 99·9 and 0·049% of the total respectively.) 2-Amino-2-deoxy-D-glucose and -D-galactose also give equilibrium mixtures of the protonated and unprotonated forms, causing oxidation of the monosaccharide by two different routes. Thus, the free 2-amino-2-deoxy hexose is oxidized by 5 moles of periodate in the normal Malapradian manner, giving no CO_2. In the protonated form, however, the ammonium group prevents cleavage of the $C_{(1)}$–$C_{(2)}$ and $C_{(2)}$–$C_{(3)}$ bonds, giving ammonium malonaldehyde which is oxidized to give 1 mole of CO_2 with a total consumption of 6 moles of periodate. Consequently the yield of CO_2 on oxidizing a 2-amino hexose will increase with increasing acidity of the reaction mixture. Thus, oxidations of 2-amino-2-deoxy-D-glucose at pH 5·0, 3·6 and 1·2 gave 0·05, 0·3 and 0·6 mole of CO_2 respectively. Quantitative evaluation of the competing pathways is made difficult by the continuous re-establishment of the equilibrium in one direction or the other according to which form is oxidized more rapidly at a given pH.

Oxidation of *N*-(2:4-dinitrophenyl)-2-amino-2-deoxy-D-glucose proceeded in a similar manner to 2-acetamido-2-deoxy-D-glucose, but at pH 5·0–3·6 a relatively stable intermediate, namely *N*-(2:4-dinitrophenyl)-glyoxylamide accumulated, causing low yields of CO_2.

REFERENCES

Cantley, M., Hough, L. & Pittet, A. O. (1959). *Chem. & Ind.* p. 1126.
McCasland, G. E. & Smith, D. A. (1951). *J. Amer. chem. Soc.* **73**, 5164.

Effects of Prolonged Oxygenation on the Cytochrome and Enzyme Systems of *Torula*

By P. B. Scutt. (*Molteno Institute, University of Cambridge, and Department of Biochemistry, University of Western Australia*)

It has been shown (Fink & Berwald, 1933) that when brewer's yeast, which is adapted to a mainly anaerobic, fermentative life, is grown under aerobic conditions it adapts to these conditions, and the cytochrome spectrum changes from the two-banded spectrum typical of brewer's yeast (Fink, 1932) to the four-banded one of baker's yeast

(Keilin, 1925). Lindenmayer & Estabrook (1958) have shown the converse process in baker's yeast grown anaerobically, and its reversal on aeration.

Chin (1950) brought about a similar change in aerated brewer's yeast under conditions not permitting multiplication, showing the change to be a transformation within the cell and not a selection process.

This paper describes the extension of such experiments to the effects of an oxygen concentration higher than atmospheric on a yeast, *Torula utilis*, normally adapted to aerobic life, and showing a typical four-banded cytochrome spectrum.

Pure oxygen was bubbled vigorously through a suspension of the yeast in a medium not supporting growth for periods up to 10 days. Glucose was supplied and diffusible metabolic products were removed by diffusion through cellophane. The cytochrome and pyridine haemochromogen spectra were observed using samples removed at intervals and the rates of aerobic oxygen uptake and anaerobic carbon dioxide production were measured in Barcroft manometers.

The only change found in the cytochromes was a progressive destruction, parallel with the general breakdown of cellular structure. At the same time the rates of respiration and of fermentation decreased. In the absence of glucose there was a similar breakdown of the cytochromes, but the decline in the rate of fermentation was much faster and that in the rate of respiration rather slower.

It is suggested that under these conditions enzymes actually functioning may be maintained at the expense of those not in use.

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REFERENCES

Chin, C. H. (1950). *Nature, Lond.*, **165**, 926.
Fink, H. (1932). *Hoppe-Seyl. Z.* **210**, 197.
Fink, H. & Berwald, E. (1933). *Biochem. Z.* **258**, 141.
Keilin, D. (1925). *Proc. Roy. Soc.* B, **98**, 312.
Lindenmayer, A. & Estabrook, R. W. (1958). *Arch. Biochem. Biophys.* **78**, 66.

The Crystallization and Properties of Hepatic Arginase of Ox, Sheep and Horse

By S. J. Bach and J. D. Killip. (*Department of Physiology, University of Bristol*)

Arginase from sheep and horse liver was highly purified and finally crystallized. The purification procedures employed were slightly modified versions of the method reported previously (Bach & Killip, 1958) for the crystallization of ox-liver arginase.

The effects of pH on the activity, K_m, and $V_{max.}$ of highly purified preparations of arginase from the above three sources were investigated. The kinetic data obtained for the three preparations were in very close agreement with one another, each giving a pH optimum at 9·2 and a K_m of approximately 7×10^{-3} M in the pH range 8·5–10·0. From the similarity of their kinetic behaviour and also from that of the effect of pH on their stability, the identity of the three enzymes was considered very probable.

The graphs obtained by plotting pK_m and log $V_{max.}$ against pH were interpreted (Dixon, 1953; Dixon & Webb, 1958) as indicating three ionizing groups with pK values of approximately 8·2, 9·3 and 10·3 at the active site of the enzyme molecule, as well as two ionizing groups, with pK values of 7·9 and 9·3, of the enzyme–substrate complex.

REFERENCES

Bach, S. J. & Killip, J. D. (1958). *Biochim. biophys. Acta*, **29**, 273.
Dixon, M. (1953). *Biochem. J.* **55**, 161.
Dixon, M. & Webb, E. C. (1958). In *Enzymes*, p. 133. London: Longmans, Green and Co.

Paper Chromatography of some 2:4-Dinitrophenylhydrazones in Acid Systems

By I. E. Bush and T. D. R. Hockaday. (*Department of the Regius Professor of Medicine, Radcliffe Infirmary, Oxford*)

The paper chromatography of the 2:4-dinitrophenylhydrazones of carbonyl compounds was described by Cavallini, Frontali & Toschi (1949) and subsequently by many others. With the exception of an unstable system with 10% acetic acid in the mobile phase (Wallgren & Nordlund, 1956), the systems used have been neutral or alkaline.

Acid systems similar to those described by Bush (1960) were investigated and compared with butanol–3% aqueous ammonia (1:1, by vol.), (BuOH/NH_3) (Cavallini *et al.* 1949) in the behaviour of some 2:4-dinitrophenylhydrazones, mostly of keto acids. The three systems used were toluene–acetic acid–water (4:3:1, by vol.) (T/A75); light petroleum (b.p. 100–120°)–toluene–acetic acid–water (10:10:17:3, by vol.) (LT11/A85): and decalin–acetic acid–water (10:9:1, by vol.) (D/A90). R_F values for a number of compounds will be given; for any compound the R_F values are in the order T/A75, LT11/A85, D/A90.

Two of the acid systems (T/A75, LT11/A85) have the advantages over BuOH/NH_3 that they run faster and that the R_F values of the *cis* and *trans* isomers of the 2:4-dinitrophenylhydrazones of some α-keto acids, notably pyruvic, are very

similar, so that the compounds form one slightly elongated spot. Stewart (1953) showed that *cis* and *trans* isomers, separated by column chromatography, give different colours with a suitable alkaline solution. The contiguity of both isomers in the apparently single spot given by the acid systems can be shown by dipping the chromatogram over the surface of N-NaOH in 80% aqueous ethanol; the *cis* isomers, with higher R_F values, show little change of colour while the *trans* isomers, which have lower R_F values, become brown. This method of developing the chromatogram enables a given spot to be identified as either a *cis* or *trans* form in systems where the isomers separate (D/A90 and $BuOH/NH_3$).

The disadvantage of the acid systems is that some compounds decompose in them; thus the 2:4-dinitrophenylhydrazone of acetoacetic acid undergoes complete decarboxylation. A small loss, of 10–15%, occurs with the 2:4-dinitrophenylhydrazones of pyruvic and α-ketoglutaric acids but quantitative colourimetric determination was possible within ±3% as the loss was proportional to the amount on the chromatogram over the range 10–60γ.

R_M values between pairs of compounds of the α-keto acid derivatives [Bate-Smith & Westall (1950), and compare Meigh (1952) for 2:4-dinitrophenylhydrazones of aldehydes and ketones] show a decrease with increasing chain length and are greater with the acid than with the alkaline systems.

REFERENCES

Bate-Smith, E. C. & Westall, R. G. (1950). *Biochim. biophys. Acta*, **4**, 427.
Bush, I. E. (1960). *Symp. Biochem. Soc.* **18**, 1.
Cavallini, D., Frontali, N. & Toschi, G. (1949). *Nature, Lond.*, **163**, 568.
Meigh, D. F. (1952). *Nature, Lond.*, **170**, 579.
Stewart, H. B. (1953). *Biochem. J.* **55**, 26P.
Wallgren, H. & Nordlund, E. (1956). *Acta chem. scand.* **10**, 1671.

The Effect of Changes in Nutritional State on the Clearing-Factor Lipase Activity of Homogenates and Extracts of Rat Adipose Tissue

By D. S. Robinson and M. R. Salaman. (*External Staff of the Medical Research Council, Sir William Dunn School of Pathology, Oxford*)

In recent studies it has been shown that when intact epididymal fat bodies from rats which have been fed their normal diet or given carbohydrate in excess of their nutritional requirements are incubated with triglyceride emulsions *in vitro*, the triglyceride is hydrolysed by the action of the tissue clearing-factor lipase. On the other hand, when the fat bodies are obtained from rats which have been fasted for 48 hr., very little hydrolysis of added triglyceride by clearing-factor lipase can be demonstrated (Hollenberg, 1959; Cherkes & Gordon, 1959; Robinson, 1960*a*, *b*). These results could be explained either by a change in the accessibility of the clearing-factor lipase or by changes in the total amount of enzyme present. The latter view is supported by the experiments reported below.

The clearing-factor lipase in rat adipose tissue is inhibited by 0·5M solutions of sodium chloride and is activated by low concentrations of heparin. Further the clearing-factor lipase of extracts of acetone powders of adipose tissue, whilst fully active on chylomicron triglyceride, only hydrolyses the triglyceride of certain artificial lipid emulsions at a slow rate, unless the emulsions are activated by a plasma high-density lipoprotein fraction (Korn & Quigley, 1955, 1957). Using these characteristic properties of the enzyme to distinguish it from other lipases in the fat bodies, the activity of the clearing-factor lipase has been studied in both aqueous homogenates and aqueous extracts of acetone powders of rat-epididymal fat. The activity is much greater (4–5 times) with preparations of tissue from animals fed their normal diet than it is with preparations from animals fasted for 48 hr.

The levels of clearing-factor lipase activity in homogenates of subcutaneous, renal and omental adipose tissue are also much higher when the tissues are obtained from fed animals than when they are obtained from fasted animals.

These results were discussed in relation to the proposed role of the clearing-factor lipase in the transport of triglyceride from the blood to the tissues (Robinson & French, 1960).

REFERENCES

Cherkes, A. & Gordon, R. S. (1959). *J. lipid Res.* **1**, 97.
Hollenberg, C. H. (1959). *Amer. J. Physiol.* **197**, 667.
Korn, E. D. & Quigley, T. W. (1955). *Biochim. biophys. Acta*, **18**, 143.
Korn, E. D. & Quigley, T. W. (1957). *J. biol. Chem.* **226**, 833.
Robinson, D. S. (1960*a*). *Biochem. J.* **74**, 25P.
Robinson, D. S. (1960*b*). *J. lipid Res.* (in the Press).
Robinson, D. S. & French, J. E. (1960). *Pharmacol. Rev.* **12**, 241.

Phosphoprotein Phosphatase in Cerebral Tissues

By S. P. R. Rose and P. J. Heald. [*Department of Biochemistry, Institute of Psychiatry, (British Postgraduate Medical Federation, University of London), Maudsley Hospital, London, S.E. 5*]

Phosphoprotein phosphatase has been studied in amphibian egg (Harris, 1946), purified from mam-

malian spleen (Hofman, 1958; Glomset, 1959) and its existence noted in brain (Feinstein & Volk, 1949). Work described here reports the partial purification of phosphoprotein phosphatase from ox brain, liberating inorganic phosphate from α-casein, and its distinction from other mammalian phosphoprotein phosphatases.

The enzyme has been extracted with water from acetone-dried ox brain and partially purified by precipitation with acetone at −5°. A 60–70% yield of material purified 10- to 15-fold is obtained. The partially purified enzyme had a pH optimum below 5·5 and K_m 1·2 mM for α-casein P. In the absence of added activators, rates of phosphate release were 30–60 μmoles of inorganic phosphorus/g. wet wt./hr. Activity was completely lost on storage at 0° for a week or during attempts at fractionation by means of $(NH_4)_2SO_4$ or by precipitation at pH 5·0. Repeated freezing and thawing led to loss of activity. Dialysis against large or small volumes of water, 0·1 M-NaCl or 0·1 M-$MgCl_2$ resulted in complete loss of activity within 24 hr., 50% of the activity being lost within 30 min. Activity was not restored by recombination of individual fractions, nor by the addition of boiled or acid-precipitated brain extracts. Although thiols did not restore activity once lost, dialysis against cysteine (20 mM) prevented loss in the case of the crude extract, but not with the more highly purified preparation. The enzyme was activated up to 100% by Mn^{2+} (mM) and Mg^{2+} (mM), and inhibited by fluoride, ethylenediaminetetra-acetate and Fe^{2+} (mM); slight inhibition occurred with similar concentrations of other heavy metals. Thiols in concentrations of up to 100 mM were without effect. The enzyme was found to be without activity against β-glycerophosphate, pyrophosphate and adenosine triphosphate and has been clearly distinguished as different from enzymes acting upon phenylphosphate.

The enzyme thus appears to be a distinct phosphoprotein phosphatase and different from that purified from ox spleen by other workers, which is stable to dialysis and ammonium sulphate, activated up to 600% by thiols, and active with pyrophosphate, phenyl phosphate and adenosine triphosphate in addition to casein.

A Medical Research Council scholarship to one of us (S.P.R.R.) is gratefully acknowledged.

REFERENCES

Feinstein, R. N. & Volk, M. E. (1949). *J. biol. Chem.* **177**, 339.
Glomset, T. A. (1959). *Biochim. biophys. Acta*, **32**, 349.
Harris, D. L. (1946). *J. biol. Chem.* **165**, 541.
Hofman, T. (1958). *Biochem. J.* **69**, 135, 139.

The Subcellular Distribution of Gangliosides in Cerebral Cortex

By L. S. Wolfe.* (*Department of Biochemistry, Institute of Psychiatry, Maudsley Hospital, London, S.E.* 5)

Gangliosides are high-molecular-weight, acidic, lipid- and water-soluble substances containing *N*-acetylneuraminic acid, which occur mainly in the grey matter of brain. Recently these substances have been shown to restore the metabolic response to electrical pulses of cerebral cortex slices that have been inhibited by keeping in cold media or by incubation with protamines and histones (McIlwain, 1960). The distribution of gangliosides in subcellular fractions of cerebral grey matter is reported here.

Subcellular fractions of guinea-pig cerebral cortex were prepared by differential centrifuging of 0·32 M-sucrose homogenates and by subfractionating further in sucrose density gradients (Whittaker, 1959). The particulate and dried supernatant fractions were then extracted with chloroform–methanol (2:1, v/v), and subjected to solvent partition as described by Folch, Lees & Sloane-Stanley (1957). The upper phases were dialysed overnight and the neuraminic acid derivatives in the extracts determined as by Long & Staples (1959) using synthetic *N*-acetylneuraminic acid as standard. Sucrose interferes with the orcinol reaction and must be removed by dialysis following extraction and partition. Dialysis of subcellular fractions before extraction resulted in complete loss of gangliosides.

It was found that all the gangliosides occurred in the particulate fractions if the microsomal fraction was separated at 10000000 *g*-min. Up to 20% of the total gangliosides appeared in the supernatant fraction when the microsomes were separated at 3000000 *g*-min. Complete recovery of additions of purified ox-brain gangliosides to homogenates was obtained but the added gangliosides were found entirely in the nuclear fraction. The total native ganglioside content was equivalent to 1·54 ± 0·28 μmoles of *N*-acetylneuraminic acid/g. wet wt. of tissue (8 experiments). Although gangliosides were extracted from all subcellular fractions, 70% of the total was found in the fractions separated at centrifugal forces above 200000 *g*-min. However, particles separating between 8000 and 450000 *g*-min. contained 80% of the total ganglioside and when layered over sucrose density gradients, 33% occurred in the layer between 0·32 M- and 0·8 M-sucrose, 56% in the layer between 0·8 M- and 1·2 M-sucrose and 11% in the particles passing through 1·2 M-sucrose. Protamine sulphate added to separated suspensions of subcellular fractions com-

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pletely prevented the extraction of gangliosides. The ability to combine with basic proteins is greatest in fractions richest in gangliosides. Ethylenediaminetetra-acetic acid and lysozyme did not alter the total extractable gangliosides. Incubation with crystalline trypsin slightly lowered the amount of gangliosides extracted. Sodium deoxycholate and lysolecithin lowered considerably the amount of gangliosides in the particulate fraction but there was no corresponding increase in the supernatant fraction.

REFERENCES

Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
Long, C. & Staples, D. A. (1959). *Biochem. J.* **73**, 385.
McIlwain, H. (1960). *Biochem. J.* (in the Press).
Whittaker, V. P. (1959). *Biochem. J.* **72**, 694.

Four Major Haemoglobins in One Individual

By A. B. Raper (*Royal Infirmary, Bristol*) and D. B. Gammack, E. R. Huehns* and E. M. Shooter (*Department of Biochemistry, University College, London, W.C.* 1)

Itano, Singer & Robinson (1959) have recently suggested that it is possible for an abnormal haemoglobin to form by the combination of the abnormal halves of two haemoglobin molecules rather than through the action of a specific mutant gene. The family in which they believe this event to occur is the one described by Smith & Torbert (1958) where haemoglobin S and another abnormal haemoglobin Hopkins-2 (Ho-2) segregate independently and in which some members show the three haemoglobins A, S and Ho-2 by conventional electrophoretic techniques. Itano *et al.* (1959) have shown that haemoglobin Ho-2 has abnormal α-chains and have postulated that the haemoglobin migrating in the position of haemoglobin A is a mixture of haemoglobin A and the doubly abnormal haemoglobin Ho-2/S ($\alpha_2^{Ho\cdot 2}\beta_2^S$). The separate existence of the latter has not yet been demonstrated. Reported here is a study of a family which confirms that doubly abnormal haemoglobins form *in vivo*.

In this family haemoglobin G and haemoglobin C segregate independently. Two of its members each possess four major haemoglobins which are haemoglobins A, G and C and a fourth migrating slower than haemoglobin C on both cellulose acetate and in starch gel. Hybridization experiments following the method of Itano *et al.* (1959), but on the microscale devised by Gammack, Huehns, Shooter & Lehmann (1960) have shown that the haemoglobin G has abnormal α-chains and confirmed that the haemoglobin C has abnormal β-chains (Hunt & Ingram, 1958). Dissociation and recombination of a mixture of the two haemoglobins G and C produced two new haemoglobin species. Since haemoglobin G has abnormal α-chains and haemoglobin C abnormal β-chains it is probable that the new species are haemoglobin A and the doubly abnormal haemoglobin G/C according to the equation:

$$2\alpha_2^G\beta_2^A + 2\alpha_2^A\beta_2^C \rightarrow \alpha_2^A\beta_2^A + \alpha_2^G\beta_2^A + \alpha_2^A\beta_2^C + \alpha_2^G\beta_2^C$$

Hb-G Hb-C Hb-A Hb-G Hb-C Hb-G/C

The two new species produced migrated at the same rate as the fastest and slowest of the four haemoglobins found *in vivo* and this therefore suggests that the slowest haemoglobin is the doubly abnormal haemoglobin G/C composed of the abnormal subunits of haemoglobin G and haemoglobin C. This has been confirmed by the formation of both haemoglobins G and C when the slowest-migrating haemoglobin was hybridized with haemoglobin A and by the absence of any new species when it was hybridized with either haemoglobin G or C.

We are indebted to Drs J. MacIver and L. E. Went and their colleagues for collecting blood samples and for the identification of the haemoglobin type in two of these. One of us (D.B.G.) wishes to acknowledge financial support from the Colonial Medical Research Committee.

REFERENCES

Gammack, D. B., Huehns, E. R., Lehmann, H. & Shooter, E. M. (1960). *Biochem. J.* **76**, 55P.
Hunt, J. A. & Ingram, V. M. (1958). *Nature, Lond.*, **181**, 1062.
Itano, H. A., Singer, S. J. & Robinson, E. (1959). In *Ciba Found. Symp., Biochemistry of Human Genetics*, p. 96. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: Churchill.
Smith, E. W. & Torbert, J. V. (1958). *Bull. Johns Hopkin Hosp.* **101**, 38.

* Beit Memorial Fellow.

Oxidation of β-Phenylethanol by Alcohol Dehydrogenase of Liver

By L. D. MacLeod. (*Burden Neurological Institute, Stapleton, Bristol*)

The alcohol dehydrogenase of liver isolated in crystalline form by Bonnichsen (1950) has been shown to have a much lower turnover number than the enzyme from yeast, and it also shows less marked substrate specificity. Hubbard & Wald (1951), following the earlier suggestion of Bliss (1949), demonstrated activity of the crystalline enzyme in the retinene–vitamin A conversion. Holzer & Schneider (1955) postulated the identity of the liver alcohol dehydrogenase with glycerol dehydrogenase described by Leuthardt & Wolf (1954).

It has now been found that under conditions

similar to those recommended by Dalziel (1957) for assaying activity of alcohol dehydrogenase preparations (0·1M-glycine buffer at pH 10) diphosphopyridine nucleotide is rapidly reduced in presence of β-phenylethanol. The maximum rate of dehydrogenation observed was just over half that of ethanol under comparable conditions. This would correspond to a turnover value of 70 moles of substrate/mole of enzyme/min., taking the value quoted by Bonnichsen (1950) for his best preparations. A direct estimate gave a result of approximately 60 moles/min., using 73000 for the molecular weight of the enzyme.

REFERENCES

Bliss, A. F. (1949). *Biol. Bull. Wood's Hole*, **97**, 221.
Bonnichsen, R. (1950). *Acta chem. scand.* **4**, 715.
Dalziel, K. (1957). *Acta chem. scand.* **11**, 396.
Holzer, H. & Schneider, S. (1955). *Klin. Wschr.* **33**, 1006.
Hubbard, R. & Wald, G. (1951). *Proc. nat. Acad. Sci., Wash.*, **37**, 69.
Leuthardt, F. & Wolf, H. P. (1954). *Helv. chim. Acta*, **37**, 1732.

Synthesis of Glycollic Acid by Spinach Chloroplasts

By I. ZELITCH*. (*Departments of Botany and Biochemistry, University of Oxford*)

The use of α-hydroxysulphonates, highly specific inhibitors of glycollic acid oxidase (Zelitch, 1957), with detached leaves has suggested that a relationship exists between glycollic acid and the carbon metabolism in sunlight (Zelitch, 1958; Zelitch, 1959). Experiments have now been carried out with preparations of spinach chloroplasts under conditions similar to those previously described for studies of $^{14}CO_2$ uptake in the light (Allen, Arnon, Capindale, Whatley & Durham, 1955; Gibbs & Calo, 1959). Less than 2% of the fixed carbon was normally present in glycollic acid and 17% in amino acids. With 0·4 mM-α-hydroxy-2-pyridinemethanesulphonate, the total CO_2 taken up by the chloroplasts was hardly affected, but the glycollic acid now accounted for 23% of the total and the amino acid fraction contained only 2·7%. Thus preparations of isolated chloroplasts synthesize glycollic acid in the light in a manner similar to that previously described for whole leaves.

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Fixation of $^{14}CO_2$ in light by chloroplast preparations that are 'broken' in a hypotonic medium is more than twice as great when ribose 5-phosphate is added to the complete reaction mixture (Whatley, Allen, Rosenberg, Capindale & Arnon, 1956). The addition of ribose 5-phosphate, which might be expected to increase the metabolic pool of 3-phosphoglyceric acid, did not affect the amount of ^{14}C found in glycollic acid in the presence of α-hydroxysulphonate. Accordingly, it is unlikely that 3-phosphoglyceric acid is a direct precursor of glycollic acid.

The suggestions of Wilson & Calvin (1955) and of Weissbach & Horecker (1955) that glycollic acid arises from an unphosphorylated 2-carbon fragment produced from pentose phosphate have been supported by experiments with 'broken' chloroplasts. Ribose 5-phosphate, uniformly labelled with ^{14}C, has been found to give rise to [^{14}C]glycollic acid in air without illumination or carbon dioxide. On the assumption that only 2 of the carbon atoms of the pentose phosphate molecule serve to produce glycollic acid, the yield in the presence of α-hydroxysulphonate was about 10% of the ribose 5-phosphate added. In the absence of inhibitor, the amount of the glycollic acid was lowered and of the amino acids increased. A generalized sequence of these reactions carried out by chloroplast preparations can be expressed as follows:

$$CO_2 \xrightarrow{\text{light}} \text{'pentose phosphate'} \rightarrow \text{glycollic acid} \xrightarrow[\alpha\text{-hydroxysulphonate}]{\frac{1}{2}O_2}$$

$$\text{glyoxylic acid} \rightarrow \text{amino acids}$$

The helpful discussions of Dr V. S. Butt, in whose laboratory these experiments were conducted, and the gift of [^{14}C]ribose 5-phosphate from Dr M. Gibbs, Cornell University, are gratefully acknowledged.

REFERENCES

Allen, M. B., Arnon, D. I., Capindale, J. B., Whatley, F. R. & Durham, L. J. (1955). *J. Amer. chem. Soc.* **77**, 4149.
Gibbs, M. & Calo, N. (1959). *Plant Physiol.* **34**, 318.
Weissbach, A. & Horecker, B. L. (1955). In *Symposium on Amino Acid Metabolism*, p. 741. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Whatley, F. R., Allen, M. B., Rosenberg, L. L., Capindale, J. B. & Arnon, D. I. (1956). *Biochem. biophys. Acta*, **20**, 462.
Wilson, A. T. & Calvin, M. (1955). *J. Amer. chem. Soc.* **77**, 5948.
Zelitch, I. (1957). *J. biol. Chem.* **224**, 251.
Zelitch, I. (1958). *J. biol. Chem.* **233**, 1299.
Zelitch, I. (1959). *J. biol. Chem.* **234**, 3077.

DEMONSTRATION

The Attachment of Proteins to Cells Tanned with Pyruvic Aldehyde

By N. R. LING. (*Area Central Laboratory, Manor Hospital, Bath*)

The increasingly wide use of indirect haemagglutination tests for the detection of agglutinins to soluble proteins has led to a need for stabilized erythrocyte suspensions. Formalized erythrocytes have been successfully prepared by a method similar to that of Csizmas (1960) depending upon a progressively increasing formalin concentration, but some lysis, distortion and spontaneous agglutination of cells are hard to avoid. Methods depending upon formalinization after attachment of protein to the cell (Fulthorpe, 1957) risk modification of the attached protein.

Superior cell preparations which are mechanically stable and resist water and hypertonic salt solutions are obtained with pyruvic aldehyde.

Pyruvic aldehyde (40% solution, L. Light and Co., 1 vol.) is added to saline (3·5 vol.) and the pH brought to approximately 7·0 with 10% sodium carbonate solution. Phosphate buffer (0·15M, pH 8·0, 0·7 vol.) and human erythrocytes (1 vol. of a 50% suspension) are then added and the mixture stored at 4° for 2 days with occasional mixing. The cells are then thoroughly washed with saline and are stable in saline containing 0·1% azide for at least 6 months.

Proteins may be attached by the Boyden tannic acid technique or more simply and economically by heating the protein plus cell suspension at low ionic strength. Proteins, which are non-specifically taken up by the cells under these conditions, are firmly attached and resist subsequent washing with physiological saline and buffers. A 10% suspension of cells (1·0 vol.) is centrifuged, the supernate discarded, the cells washed once in dilute buffer [saline–water–phosphate buffer (0·15M, pH 6·0), 10:10:1, by vol.] and suspended in the same buffer containing 0·8 mg./ml. of protein antigen (0·6 vol.). After heating at 50° for 1 hr. the cells are centrifuged, washed 3 times in saline containing bovine-serum albumin (100 ml. of saline + 0·1 ml. of 20% albumin) and finally suspended in 0·2 vol. of a solution prepared by adding sodium azide (0·1 g.) and 20% bovine-serum albumin (4 ml.) to saline (100 ml.). 'Stabilized' erythrocytes with human or bovine γ-globulin or thyroglobulin attached are stable for at least 3 months at 4°. Portions may be removed and diluted in buffer as required.

REFERENCES

Csizmas, L. (1960). *Proc. Soc. exp. Biol., N.Y.*, **103**, 157.
Fulthorpe, A. J. (1957). *J. Hyg., Camb.*, **55**, 382.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **398th Meeting** *of the Society was held in the Department of Biochemistry, University of Glasgow, on Thursday and Friday,* 29 *and* 30 *September* 1960, *when the following papers were read:*

COLLOQUIUM ON 'THE BIOSYNTHESIS OF NUCLEIC ACIDS'

Abbreviations

The following abbreviations for nucleotides are used without further definition:

AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate

d (deoxy), C (cytidine), G (guanosine), T (thymidine) and U (uridine) are used similarly as prefixes

DNA	deoxyribonucleic acid
DPN	diphosphopyridine nucleotide
FAD	flavinadenine dinucleotide
RNA	ribonucleic acid

COLLOQUIUM

Chemical Aspects of Polynucleotide Synthesis.

By A. M. Michelson. (*Arthur Guinness, Son and Co. Ltd., Dublin, Ireland*)

The polymerization of nucleoside-2′,3′ cyclic phosphates can be effected by the action of a number of acid anhydrides, an intermediate fully esterified nucleotide anhydride being formed (Michelson, 1959). A survey of such reagents together with an investigation of the effect of various solvents (Letters & Michelson, 1960) has shown that in certain cases the same mixed anhydride under different conditions can give rise to polymerization or to phosphorylation (or acylation). A number of nucleotide analogue polymers have been chemically synthesized by this approach (Letters & Michelson, 1960) in addition to oligonucleotides with different base sequences and homopolymers of all the major ribonucleotides. These analogues include polymers derived from *N*′-methyluridine, 5′-thiouridine (via 5′-*O*-methanesulphonyluridine-2′,3′ cyclic phosphate) and from 5-chloro-, 5-bromo- and 5-iodo-uridine.

Though of importance at higher levels of macromolecular structure and conformation (e.g. bonding between different chains or between bases in a single helix), hydrogen bonding is of little (if any) significance with respect to the interplanar interactions leading to the stacking of bases in oligonucleotides or to the resultant effects on their ultraviolet absorption, as confirmed by the properties of oligonucleotides derived from 6-dimethylaminopurine.

While mixed anhydrides of nucleoside 5′-phosphates and other acids are of somewhat limited value for the purpose of chemical polymerization, such intermediates are extremely useful for the synthesis of nucleotide coenzymes by controlled nucleophilic displacement (anion exchange). In this way a large number of 5′-nucleotide anhydrides (including ADP, ATP, adenosine 5′-tetraphosphate, FAD, UDPglucose, TDPglucose and coenzyme A) have been synthesized easily and rapidly in good yield. The methods have also been applied to the total synthesis of peptide–oligonucleotide anhydrides and of dinucleotides containing a terminal 5′-pyrophosphate group. These latter are of possible interest in connexion with the action of polynucleotide phosphorylase.

Various chemical aspects of this work are of relevance to the enzymic polymerization of nucleotides.

Letters, R. & Michelson, A. M. (1960). *J. chem. Soc.* (in the Press).
Michelson, A. M. (1959). *J. chem. Soc.* pp. 1371, 3655.

Mode of Action of Polynucleotide Phosphorylase.

By Marianne Grunberg-Manago. (*Institut de Biologie Physico-Chimique, Paris, France*)

Current studies of the assays, kinetics, substrate specificity, cofactors and primers of polynucleotide phosphorylase (PNP) purified from several sources have served to define questions of reaction mechanism:

(1) *The nature and significance of an orthophosphate–nucleotide diphosphate exchange reaction.* In yeast, unlike *Azotobacter vinelandii* or *Escherichia coli*, this process can be separated from the enzyme catalysing polymer formation and phosphorolysis.

(2) *The role of* Mg^{2+} *in the catalysis and equilibrium of formation of polyadenylic acids.* Mg^{2+} or ADP in excess both lower the rate of polymerization. The equilibrium is affected by the amount of Mg^{2+}.

(3) *The action of polyribonucleotides as primers in the polymerization reaction of ADP and adenine–guanine–uracil–cytosine polymer by a highly purified PNP.* The priming ability of different samples of RNA for polymerization were compared. The

molecular weights of polyadenylic acids formed are not markedly affected by the presence of primer.

(4) *The inability of PNP to phosphorolyse soluble RNA.* Soluble RNA from different organisms, and fractions of soluble RNA of yeast (purified on a column of diethylaminoethylcellulose), are phosphorolysed to the extent of 20–30%, whereas all the other samples of RNA are almost completely phosphorolysed.

(5) *Influence of amino acids on PNP* in vivo. In an effort to examine this problem in a system closely approximating to physiological conditions *in vivo*, studies were carried out on cell suspensions exposed to toluene. This system provided a reliable assay for PNP. Employing toluene-treated suspensions of a tryptophan-requiring mutant of *E. coli* (which also contain levels of chloramphenicol sufficient to prevent protein synthesis), it has been found that the specific activity of PNP and the amount of RNA synthesized are both a function of added tryptophan. Tryptophan can be replaced by DL-5-methyltryptophan, but a large number of other naturally occurring amino acids have little or no effect.

Studies on the Biosynthesis of Ribonucleic Acid.

By R. H. BURDON. (*Department of Biochemistry, The University, Glasgow, W.* 2)

A soluble system has been obtained from Ehrlich ascites tumour cells which is capable of synthesizing RNA (Burdon & Smellie, 1960*a*, *b*). On fractionation with ammonium sulphate this yields two fractions (*A* and *B*) capable of catalysing the incorporation of ^{32}P-labelled uridine 5′-triphosphate (U^{32}P-P-P) into RNA. Both fractions require Mg^{2+} and are inactivated by heating to 50°.

The incorporation of labelled UTP into RNA by fraction *A* is optimal at pH 9·5 and requires reduced DPN. The reaction is stimulated by the addition of a mixture of ATP, GTP and CTP and is inhibited by inorganic pyrophosphate and to a lesser extent by orthophosphate. In the presence of ATP, GTP, CTP and labelled UTP a net increase of about 12% in the amount of polyribonucleotide per reaction vessel can be detected over the incubation period (10 min. at 37°). Studies on the distribution of ^{32}P in alkaline hydrolysates of the RNA indicate that when ATP, GTP and CTP are omitted from the reaction mixture a high proportion of the uridine moieties are present as polyuridylic acid whereas in the presence of the other three nucleoside triphosphates there is a more random distribution of the incorporated uridine moiety.

The optimum pH for the incorporation of labelled UTP by fraction *B* is 7·7. This system is slightly inhibited by the addition of a mixture of ATP, GTP and CTP is strongly inhibited by inorganic pyrophosphate and is unaffected by inorganic orthophosphate. No net synthesis of polynucleotide can be demonstrated with this fraction.

Burdon, R. H. & Smellie, R. M. S. (1960*a*). *Biochem. J.* **76**, 2P.

Burdon, R. H. & Smellie, R. M. S. (1960*b*). *Biochem. J.* **76**, 21P.

Pyrophosphorolysis of Soluble Ribonucleic Acid.

By E. S. CANELLAKIS. (*Department of Pharmacology, Yale University School of Medicine, New Haven, Conn., U.S.A.*)

In the presence of soluble RNA and the ribonucleotide-incorporating enzyme, ^{32}P-labelled inorganic pyrophosphate caused the pyrophosphorolysis of the soluble RNA with the consequent liberation of the ^{32}P-labelled ribonucleoside 5′-monophosphates of adenine, guanine, cytosine and uracil (Canellakis & Herbert, 1960). This has been further established by incubating non-radioactive inorganic pyrophosphate under the same conditions as above with ^{32}P-labelled soluble RNA.

A large part of the action of inorganic pyrophosphate can be shown to be exerted on the terminal sequence of the soluble RNA. Native soluble RNA and pyrophosphorylized soluble RNA have been incubated with ^{32}P-labelled CTP (C^{32}P-P-P) and the distribution of the ^{32}P on the ribonucleotides has been determined after alkaline hydrolysis of the isolated soluble RNA. It has been found that the ^{32}P is found to be predominantly attached to CMP-2′(3′) when native soluble RNA is the substrate and to be equivalently attached to CMP-2′(3′) and to AMP-2′(3′) when the pyrophosphorylized soluble RNA is the substrate. Evidence was presented to support the conclusion that adenylic acid is the fourth ribonucleotide in at least a large portion of soluble RNA, forming the terminal sequence A-C-C-A-(soluble RNA).

Canellakis, E. S. & Herbert, E. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 170.

Effect of Phosphodiesterase on the Capacity of Soluble Ribonucleic Acid to Accept Amino Acids.

By EDWARD HERBERT. (*Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass., U.S.A.*)

The enzymes which incorporate 5′-ribonucleotides into soluble RNA have been fractionated

from rat liver along with the soluble RNA into three ribonucleoproteins which have been designated α, β, and γ. Each of these ribonucleoproteins has been fractionated further into the enzyme component and the soluble RNA component. Evidence was presented to show that these soluble RNA components can be degraded with snake-venom phosphodiesterase to the point where their capacity to accept amino acids (a biological property of the RNA) is lost and that, following incubation of the diesterase-degraded soluble-RNA chains with ribonucleoside triphosphates and purified nucleotide-incorporating enzymes, their capacity to accept amino acids is restored. Evidence was also presented to support the contention that the phosphodiesterase degrades the soluble RNA in random fashion and that reincorporation of ribonucleotides into the degraded soluble RNA is actually responsible for the reconstitution of the capacity of this material to accept amino acids.

Some Characteristics of the Phosphorylating Kinases of Neoplastic and Normal Cells.

By P. Bianchi, J. A. V. Butler, A. R. Crathorn and K. V. Shooter. (*Chester Beatty Research Institute, Fulham Road, London, S.W. 3*)

The phosphorylation of thymidine and subsequent synthesis of DNA have been studied using tritiated thymidine. There are marked differences in the activity of phosphorylating kinases in supernatant fractions derived from normal and leukaemic spleens, the latter being more active by a factor of about 10. In all cases the amount of diphosphates present is small, but the proportions of mono- to triphosphate depend on the time of incubation, the triphosphate predominating with the longer times. In contrast the overall rates of synthesis of DNA in the two cases differ only by a factor of 2.

The Ehrlich mouse ascites cells have activities similar to leukaemic spleens, while the M.1 rat sarcoma yields supernatants similar to normal spleen. Normal rat-liver supernatant does not produce a significant inhibition of activity when added to leukaemic spleen supernatant. The Ehrlich ascites cells, incubated *in vitro*, accumulate thymidine in their nuclei but little is found in the cytoplasm. In a salt medium accumulation in the nucleus and incorporation into DNA ceases after only 1 hr., but in a medium containing 50% horse serum, with 50% of the salt medium, both processes are still continuing after 4 hr. These processes (mainly anaerobic) are insensitive to irradiation. It would appear to be necessary to assume a stage at which the phosphorylating kinases as well as the polymerizing enzyme are present in the nucleus.

Experiments to study this have been carried out with rat regenerating liver. In 24 hr. or 30 hr. supernatants, phosphorylation stops at the monophosphate stage. This is not due to an inhibitor of the further stages because the mixing of this supernatant with that from leukaemic spleen does not prevent the formation of triphosphate. Since at this time DNA synthesis is maximal, it is clear that the necessary enzymes must be present somewhere within the cell and experiments have been performed to demonstrate their existence within the nucleus prior to the onset of synthesis of DNA.

Studies on the Biosynthesis of Deoxyribonucleic Acid.

By R. M. S. Smellie. (*Department of Biochemistry, The University, Glasgow, W. 2*)

The biosynthesis of DNA has been examined in extracts of Ehrlich ascites tumour cells, normal and regenerating rat liver and in disrupted preparations of L strain fibroblasts grown in tissue culture (Smellie, Gray, Keir, Richards, Bell & Davidson, 1960; Weissman, Gray, Thomson, Smellie & Davidson, 1960). The process involves the phosphorylation of the 5′-monophosphates dAMP, dGMP, dCMP, and TMP to the corresponding triphosphates, dATP, dGTP, dCTP and TTP, by a series of kinases, and the subsequent polymerization of these to form polynucleotide under the influence of a polymerase.

The levels of activities of the kinases for the formation of dATP, dGTP and dCTP are the same in normal and regenerating rat liver but thymidine, TMP and TDP kinases are not detectable in extracts of normal liver and appear sequentially in regenerating liver in the order, thymidine, TMP and TDP kinase between 18 and 40 hr. after partial hepatectomy. Similar observations have been made in cultures of L strain fibroblasts. Thus TMP and TDP kinase activities are very low in extracts of cells harvested shortly after inoculation, rise to a peak during the period of rapid growth and decline before the completion of growth of the culture.

The polymerase activity of normal liver is also very low while that of regenerating liver is high. When extracts of normal and regenerating liver are mixed there is marked inhibition of polymerase, thymidine and thymidylate kinases but not of dAMP, dGMP or dCMP kinases. Similar inhibition of polymerase and thymidylate kinases from Ehrlich ascites tumour cells and bone marrow has been observed with extracts of normal liver and kidney. Fractionation of normal liver extracts has yielded separate fractions, one of which inhibits the kinases and the other the polymerase. Both inhibitory factors are heat labile and non-diffusible.

The mechanisms of these inhibitions and their

importance in relation to the control of the biosynthesis of DNA were discussed.

Smellie, R. M. S., Gray, E., Keir, H. M., Richards, J., Bell, D. & Davidson, J. N. (1960). *Biochim. biophys. Acta*, **37**, 243.

Weissman, S., Gray, E., Thomson, R. Y., Smellie, R. M. S. & Davidson, J. N. (1960). *Biochem. J.* **76**, 26P.

Biosynthesis of Bacteriophage Deoxyribonucleic Acid.

By LISELOTTE I. FESSLER. (*Department of Biochemistry, University of Oxford*)

This paper reviewed the literature on the change in the enzymes involved in the synthesis of nucleic acids in *Escherichia coli* infected with T-even bacteriophages. Following infection the synthesis of bacterial DNA ceases and after an initial lag period bacteriophage DNA is formed. During this early period of infection enzymes are formed which appear to control the rate of synthesis and the type of DNA formed. Thus, in the T-even bacteriophages, where cytosine is replaced by hydroxymethylcytosine, enzymes appear for the formation of the precursor deoxyhydroxymethylcytidinetriphosphate, for the removal of deoxycytidinetriphosphate, and for the attachment of glucose to the hydroxymethylcytosine of the DNA. There are also increases in the activities of several other enzymes.

These enzymes begin to be formed before bacteriophage DNA is synthesized and the question arises how this change in enzyme pattern is initiated. During the early period of infection a new type of RNA is formed and with the use of the mutant *E. coli* B94, requiring both adenine and arginine, Volkin (1960) reported evidence that RNA formed at this time has biological activity.

Volkin, E. (1960). *Fed. Proc.* **19**, 305.

COMMUNICATIONS

(1) Phosphorylation of Some Analogues of Fructose by Yeast Hexokinase.

By J. R. BURT.* (*Department of Physiology and Biochemistry, The University, St Andrews, Fife*)

Yeast hexokinase catalyses the phosphorylation of a variety of sugars and structurally related compounds (Sols, Fuente, Villar-Palasi & Asencio, 1958). Some of these substrates are furanoid, but most are pyranoid. Since the majority of compounds that have been tested are analogues of glucopyranose, four analogues of fructofuranose were synthesized and tested for substrate and inhibitor activity using the hexokinase preparation of Gamble & Najjar (1955) and a modification of the spectrophotometric indicator assay method of Crane & Sols (1955).

1-Deoxy-D-fructose was prepared from 1-deoxy-2:3-4:5-di-*O*-*iso*propylidene-1-thioethyl-D-fructose (Baker, 1955) by treatment with Raney nickel followed by acid hydrolysis. 1-Deoxy-D-fructose is phosphorylated ($K_m = 1{\cdot}09 \times 10^{-2}$M) and inhibits the phosphorylation of fructose by yeast hexokinase.

2:5-Anhydro-D-mannitol was prepared from 2:5-anhydro-D-mannose (Grant, 1956) by hydrogenation with Raney nickel or sodium borohydride (Bera, Foster & Stacey, 1956). This compound is phosphorylated by hexokinase and appears to inhibit its own phosphorylation.

1-Amino-1-deoxy-D-fructose (Kuhn & Haas, 1956) is not phosphorylated, but inhibits phosphorylation of glucose. 2:5-Anhydro-D-sorbitol (Hockett, Zief & Goepp, 1946) is neither a substrate nor an inhibitor for yeast hexokinase.

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Baker, S. B. (1955). *Canad. J. Res.* **33**, 1459.

Bera, B. C., Foster, A. B. & Stacey, M. (1956). *J. chem. Soc.* p. 4531.

Crane, R. K. & Sols, A. (1955). In *Methods in Enzymology*, vol. 1, p. 279. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Gamble, J. L., jr & Najjar, V. A. (1955). *J. biol. Chem.* **217**, 595.

Grant, A. B. (1956). *N.Z. J. Sci. Tech.* **37**B, 509.

Hockett, R. C., Zief, M. & Goepp, R. M. (1946). *J. Amer. chem. Soc.* **68**, 935.

Kuhn, R. & Haas, H. J. (1956). *Liebigs Ann.* **600**, 148.

Sols, A., Fuente, G. de la, Villar-Palasi, C. & Asencio, C. (1958). *Biochem. biophys. Acta*, **30**, 92.

(2) The Nature of 'Cholesterol' in normal Human-Blood Plasma.

By MARION GREIG and R. P. COOK. (*Department of Biochemistry, Queen's College, Dundee, University of St Andrews, Scotland*)

The reported values for cholesterol (cholest-5-en-3β-ol) in human-blood plasma (as % of total sterol, calculated as Liebermann–Burchard chromogens and/or digitonin-precipitable compounds) vary from 99 (Abell, Levy & Kendall, 1952) to about 66 (Monasterio & Berti, 1952). The sterols present in pooled normal human-blood plasma (citrated) were estimated when possible by gravimetric methods. The ethanol–diethyl ether (2:1, v/v) extract was separated by chromatography on

silicic acid–Celite into the ester and free sterol fractions. The ester fraction was saponified and the sterol recovered, analyses being made of the fatty acids. The combined sterol fractions were treated with oxalic acid to give an adduct which was decomposed and the sterol crystallized from acetic acid (Fieser, 1953). The yield of pure cholesterol was 90% of the isolated sterol fractions. 'Fast-acting' sterols formed about 2% (but no detectable 7-dehydrocholesterol at a concentration of 10 μg./ml.). Stanols, i.e. non-chromogens which are digitonin-precipitable, were about 5%. A chromogen fraction not digitonin-precipitable was observed. A negative discrepancy of about 20% was found between values for 'cholesterol' estimated by the method of Sperry & Webb (1950) on small samples and the balance obtained by the gravimetric methods. Cholesterol is the major but not the sole sterol in human-blood plasma.

Abell, L. I., Levy, B. B. & Kendall, F. E. (1952). *J. biol. Chem.* **195**, 357.
Fieser, L. F. (1953). *J. Amer. chem. Soc.* **75**, 4395.
Monasterio, G. & Berti, O. (1952). *Klin. Wschr.* **30**, 111.
Sperry, W. M. & Webb, M. (1950). *J. biol. Chem.* **187**, 97.

(3) Inhibition of Incorporation of ^{14}C-Labelled Purines into Nucleic Acids by a Substituted Pyrimidine.

By B. A. Newton. (*Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Antrycide (2:6′-dimethyl-6:4′-pyrimidylamino-4:2′-diaminoquinoline 1:1′-dimethosulphate) has been shown to inhibit the incorporation of [^{14}C]-adenine and [^{14}C]guanine into nucleic acid of the trypanosomid flagellate *Strigomonas oncopelti* (Newton, 1958). The net synthesis of nucleic acids or the incorporation of [^{14}C]glycine, [^{14}C]uracil or ^{32}P into them were, on the other hand, shown to be much less affected; thus, 5 μM-antrycide caused a 50% inhibition of incorporation of adenine without inhibiting incorporation of uracil and 100 μM-antrycide caused a 90% inhibition of incorporation of adenosine with only a 30% inhibition of incorporation of uracil. The inhibition of incorporation of adenine was unaffected by the ratio of purine to drug. Further investigation has revealed that it is the pyrimidine portion of the antrycide molecule which is responsible for the inhibition of incorporation of purine, the quinoline portion being completely inactive in concentrations up to mM. Tests carried out on a number of substituted pyrimidines have led to the conclusion that a quaternary nitrogen atom in the pyrimidine ring is essential for inhibitory activity.

Attempts have been made to determine the point at which antrycide inhibits the incorporation of purines into nucleic acids. Using washed cell suspensions of *Strigomonas oncopelti* it has been shown that the drug has no effect on the transfer of [^{14}C]adenine from the external environment into the intracellular 'nucleotide pool'. Column chromatography (Dowex-1, Cl^-) of acid-soluble material obtained from control and drug-treated organisms showed that the relative amounts of adenine, adenosine and AMP, ADP and ATP present in the cells were unchanged by drug treatment, also that the distribution of ^{14}C in these compounds was unaffected by the drug. These results suggest that antrycide does not affect the synthesis of nucleotides from purine bases but may interfere at some point in the polymerization of acid-soluble nucleotides to nucleic acids. Polynucleotide phosphorylase is known to catalyse the polymerization of nucleoside 5′-diphosphates *in vitro* with the release of orthophosphate (Grunberg-Manago, Ortiz & Ochoa, 1956) and it was postulated that this enzyme may be involved in the synthesis of RNA *in vivo*. A partially purified preparation of this enzyme from *Strigomonas oncopelti* was not inhibited by 200 μM-antrycide.

Grunberg-Manago, M., Ortiz, P. J. & Ochoa, S. (1956). *Biochim. biophys. Acta*, **20**, 269.
Newton, B. A. (1958). *J. gen. Microbiol.* **19**, ii.

(4) Sites of Synthesis of a Nucleic Acid and a Protein in Infected Krebs-2 Ascites Tumour Cells.

By A. J. D. Bellett and A. T. H. Burness. (*Virus Research Unit, Medical Research Council Laboratories, Carshalton, Surrey*)

Murine encephalomyocarditis virus is a small animal virus probably composed of a ribonucleoprotein core surrounded by a coat consisting of a number of discrete protein sub-units. Krebs-2 mouse ascites tumour cells infected with this virus *in vitro* synthesize an infective RNA component (Huppert & Sanders, 1958) and also a specific protein (haemagglutinin) (Sanders, Huppert & Hoskins, 1958). The RNA can be extracted from infected cells using cold phenol and its infectivity may then be estimated. The protein may be estimated by its ability to agglutinate red-blood cells. These characteristic properties have been used to study the sites of synthesis of the specific nucleic acid and protein in sub-cellular fractions of Krebs-2 cells infected with the virus.

Infective nucleic acid is first detectable in the nuclei 30 min. after infection at 4°. The concentration in this fraction rises during incubation at 37°

reaching a maximum 4·5 hr. after infection, a 70-fold increase taking place between 3 and 4·5 hr. There is a small increase in the infectivity of RNA extracted from the mitochondrial fraction during this time. After 4·5 hr. the infectivity of RNA in the nuclear fraction decreases, the loss being accompanied by an increase in the infectivity of RNA from the mitochondrial fraction. After about 6 hr. there is a decrease in the infective RNA associated with the mitochondrial fraction, presumably due to incorporation into virus from which it cannot be extracted by cold phenol (Huppert & Sanders, 1958).

The virus surface protein, or haemagglutinin, is first detected in high concentration in the mitochondrial fraction at 4·5 hr. although the nuclear fraction also contains haemagglutinating material at this time. The haemagglutinin content of the mitochondrial fraction rises rapidly between 5 and 6 hr. after infection but the haemagglutinin content of the nuclear fraction does not rise to the same extent. Between 6 and 8 hr. after infection there is an increase in the amount of haemagglutinin depositing with the microsomes.

The significance of these results was discussed.

Huppert, J. & Sanders, F. K. (1958). *Nature, Lond.*, **182**, 515.

Sanders, F. K., Huppert, J. & Hoskins, J. M. (1958). *Symp. Soc. exp. Biol.* **12**, 123.

(5) Mechanism of Conjugation of *o*-Aminophenol in Plants and an Insect.

By G. J. Dutton and Anne M. Duncan. (*Department of Biochemistry, Queen's College, Dundee, University of St Andrews, Scotland*)

Mammalian tissues form glucuronides by glucuronyl transference from UDPglucuronic acid to acceptors, utilizing UDP-transglucuronylase (see Dutton, 1960).

Simple glucuronides exist in few plants: e.g. chrysin and quercetin glucuronides in *Scutellaria* and *Phaseolus vulgaris* respectively, and a possible glucosylglucosiduronic acid conjugate in sugar beet (see Levvy & Marsh, 1959).

Before studying conjugation of natural aglycones in these plants, that of *o*-aminophenol was followed. Beet-tuber slices formed a conjugate behaving like *o*-aminophenyl glucuronide in Levvy & Storey's (1949) method, but not hydrolysable by β-glucuronidase. Corresponding homogenates synthesized it in presence of UDPglucose or a system producing this (e.g., ATP + UTP + glucose or glucose 1-phosphate), but not of UDPglucuronic acid. ATP + UTP produced some synthesis (helped by endogenous glucose?), not increased with glucuronate and decreased with glucurone or galacturonate. UDP-glucuronic acid did not affect conjugation here due to UDPglucose.

Presumably the glucoside was being synthesized by the mechanism known elsewhere in plants (Cardini & Leloir, 1957; Jacobelli, Tabone & Tabone, 1958). Plant glucuronyl transfer to simple phenols has not been shown (Yamaha & Cardini, 1960*a*, *b*) and preliminary experiments indicated no glucuronyl transference to *o*-aminophenol in *Scutellaria galericulata*. During this work, C. A. Marsh (personal communication) reported that, although quercetin β-D-glucosiduronic acid is formed by glucuronyl transfer from UDP-glucuronic acid in *Phaseolus vulgaris*, other acceptors are not utilized. Even plants able to form simple glucuronides may therefore 'detoxify' administered phenols through glucoside formation (see Hutchinson, Roy & Towers, 1958; Pridham & Saltmarsh, 1960).

Insects also form glucosides rather than glucuronides (Smith, 1955). *Periplaneta americana* hepatic caecum homogenates conjugated *o*-aminophenol when UDPglucose, or ATP + UTP + glucose 1-phosphate were added; UDPglucuronic acid was ineffective and the conjugate, not hydrolysable by β-glucuronidase, was partially destroyed by crude β-glucosidase. Conjugation was greater at 25° than at 37°.

Cockroach hepatic caecum therefore contains enzymes (i) transferring glucose from UDPglucose, but not glucuronic acid from UDPglucuronic acid, to *o*-aminophenol; (ii) probably forming UDP-glucose from ATP + UTP + glucose 1-phosphate. Candy & Kilby (1960) demonstrated UDPglucose donating glucose for synthesis of trehalose in locusts, and the above results indicate its role in detoxication among the Insecta.

Though the much-used Levvy & Storey (1949) method cannot distinguish between *o*-aminophenyl glucuronide and glucoside, adequate controls are simple, and the glucoside was not formed in mammals.

Candy, D. J. & Kilby, B. A. (1960). *Biochem. J.* **74**, 19P.

Cardini, E. E. & Leloir, L. F. (1957). *Cienc. e Invest.* **13**, 514.

Dutton, G. J. (1960). *Biochem. Pharmacol.* (in the Press.)

Hutchinson, A., Roy, C. & Towers, G. H. N. (1958). *Nature, Lond*, **181**, 841.

Jacobelli, G., Tabone, M. J. & Tabone, D. (1958). *Bull. Soc. Chim. biol., Paris*, **40**, 955.

Levvy, G. A. & Marsh, C. A. (1959). *Advanc. Carbohyd. Chem.* **14**, 381.

Levvy, G. A. & Storey, I. D. E. (1949). *Biochem. J.* **44**, 295.

Pridham, J. B. & Saltmarsh, M. J. (1960). *Biochem. J.* **74**, 42P.

Smith, J. N. (1955). *Biol. Rev.* **30**, 455.

Yamaha, T. & Cardini, C. E. (1960*a*). *Arch. Biochem. Biophys.* **86**, 127.
Yamaha, T. & Cardini, C. E. (1960*b*). *Arch. Biochem. Biophys.* **86**, 133.

(6) Mechanism of Glucuronide Synthesis in Skin.

By I. H. Stevenson and G. J. Dutton. (*Department of Biochemistry, Queen's College, Dundee, University of St Andrews, Scotland*)

Glucuronide synthesis in skin is important both from the presence there of uronic acid-containing mucopolysaccharides and from the conjugation in that tissue of various foreign substances; e.g. the carcinogen 3:4-benzopyrene is metabolized there through benzopyrenols to the soluble benzopyrenyl glucuronides (see Harper & Calcutt, 1960).

Conjugation of *o*-aminophenol has been studied in skin, and the mechanism of glucuronide formation appears so far to be similar to that shown in liver and gastro-intestinal tract (Storey & Dutton, 1955; Dutton & Stevenson, 1959).

As suggested by Zini (1952) a conjugate was formed when strips of shaved (adult) mouse or (foetal and infant) guinea-pig skin were incubated aerobically with *o*-aminophenol. This was hydrolysable by β-glucuronidase unless saccharolactone, inhibiting this enzyme, was also present. Nitrogen-gassed skin homogenates from infant mice or guinea pigs synthesized the conjugate only when UDPglucuronic acid, and not UDPglucose, was added.

Optimal conditions in homogenates have not yet been achieved but, with mouse whole-skin strips, 20 mM-glucose doubled the rate of synthesis and levels of 1·0–1·6 μg. of *o*-aminophenol conjugated/50 mg. dry wt. tissue/hr. were obtained. Incubation prolonged to 6 hr. produced little variation in rate.

Evidence of UDPglucuronic acid in boiled extracts of mouse and guinea-pig skin was obtained but guinea-pig-skin homogenates, unlike similar preparations from liver and gastro-intestinal tract, have not so far shown UDPglucose dehydrogenase activity: incubation of UDPglucose with diphosphopyridine nucleotide under oxygen yielded no detectable UDPglucuronic acid. However, the latter is rapidly destroyed in skin homogenates (see also Pogell & Krisman, 1960); a notable increase in *o*-aminophenyl glucuronide formation on addition of ATP to UDPglucuronic acid-fortified mouse-skin homogenates, gassed with nitrogen, may be due to inhibition of the responsible pyrophosphatases, although the possibility of an 'alternative pathway' of glucuronide formation is being investigated. Interfering measurement of *o*-aminophenyl sulphate is considered unlikely. Work is also continuing on the distribution of glucuronide synthesis within whole skin and during development.

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Dutton, G. J. & Stevenson, I. H. (1959). *Biochim. biophys. Acta*, **31**, 568.
Harper, K. H. & Calcutt, G. (1960). *Nature, Lond.*, **186**, 80.
Pogell, B. M. & Krisman, C. R. (1960). *Biochim. biophys. Acta*, **41**, 349.
Storey, I. D. E. & Dutton, G. J. (1955). *Biochem. J.* **59**, 279.
Zini, F. (1952). *Sperimentale*, **102**, 40.

(7) Transfer of Soluble Ribonucleic Acid to Microsomes in an *in vitro* System from Rat Liver.

By C. J. Smith and E. Reid. (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W.* 3)

The hypothesis that soluble RNA is a precursor of microsomal RNA *in vivo* (Reid & Stevens, 1957, 1958) has been tested with an *in vitro* system.

Sucrose homogenates were prepared from the livers of rats injected with [6-^{14}C]orotic acid and were centrifuged at 105 000 *g* for 90 min. Ribonucleoprotein was prepared from the supernatant fraction, usually by repeated precipitation at pH 5·2. This ribonucleoprotein was incubated at pH 7·4, with unlabelled microsomes, prepared by centrifuging at 105 000 *g*, ATP, GTP, Mg^{2+} and an ATP-generating system. After incubation, particulate material was isolated either (i) by centrifuging at 105 000 *g* for 90 min., or (ii) by addition of calcium chloride followed by centrifuging at 10 000 *g* for 10 min. (Schneider, 1946).

Using method (i) transfer of radioactivity into microsomal RNA was demonstrable, but diminished with incubation times exceeding 5 min. Omission of GTP lowered the transfer observed. If the complete system was maintained at 0°, a spontaneous, but smaller transfer of label occurred. These results agree with those of Hoagland (1958*a*, *b*) and of Hultin & Von der Decken (1958, 1959), although not with those of Bosch, Bloemendal & Sluyser (1959, 1960) who found no requirement for GTP. Liver taken 48 hr. after partial hepatectomy was found to behave similarly to normal liver.

Material isolated after incubation, by method (ii), showed uptake of radioactivity similar to that of microsomes sedimented at 105 000 *g*. However, incorporation continued for 20 min. of incubation, by which time over half of the added label had been transferred. Replacement of ATP and GTP by sodium pyrophosphate of the same molarity gave a much lowered transfer. When labelled product obtained by method (ii) was extracted with oxalate

solution (Reid & Stevens, 1958) the residue showed more marked transfer than the extract.

The experiments suggest that with the complete system, in addition to some spontaneous transfer of labelled soluble RNA to microsomes, there is an active incorporation. This may be accompanied by conversion of microsomal RNA into a form which is not totally sedimentable at 105 000 *g*, but is still precipitable by calcium chloride.

Bosch, L., Bloemendal, H., Sluyser, M. (1959). *Biochim. biophys. Acta*, **34**, 272.
Bosch, L., Bloemendal, H. & Sluyser, M. (1960). *Biochim. biophys. Acta*, **41**, 444, 454.
Hoagland, M. B. (1958*a*). In *Proceedings of the IVth International Congress of Biochemistry*, vol. VIII, p. 199. London: Pergamon Press.
Hoagland, M. B. (1958*b*). *Rec. Trav. chim. Pays-Bas*, **7**, 631.
Hultin, T. & Von der Decken, A. (1958). *Exp. Cell Res.* **15**, 254.
Hultin, T. & Von der Decken, A. (1959). *Exp. Cell Res.* **16**, 444.
Reid, E. & Stevens, B. M. (1957). *Biochem. J.* **67**, 262.
Reid, E. & Stevens, B. M. (1958). *Nature, Lond.*, **182**, 441.
Schneider, W. C. (1946). *J. biol. Chem.* **166**, 595.

(8) *p*-Hydroxyphenylacetic acid *O*-sulphate: a Metabolite of Tyrosine *O*-sulphate in the Rat.

By K. S. Dodgson, Gillian M. Powell, F. A. Rose and N. Tudball. (*Department of Biochemistry, University College, Cardiff, and Rheumatism Research Centre, University of Manchester*)

Recently, Dodgson, Powell & Tudball (1960) showed that tyrosine *O*-sulphate (TOS), a component of some mammalian fibrinogens, is metabolized by the rat without appreciable loss of ester sulphate. Paper chromatography of the urines of rats injected intraperitoneally with TO^{35}S showed the presence of two major metabolites together with small amounts of unchanged starting material.

One of the metabolites of TOS has now been identified as the *O*-sulphate ester of *p*-hydroxyphenylacetic acid. Rats were injected intraperitoneally with TO^{35}S at a dose level of 1·5 mg./200 g. body wt. and the first urines to be voided were collected and pooled. *p*-Hydroxyphenylacetic acid *O*-sulphate was separated by the following experimental sequence: passage through Dowex 50 resin (H^+) and collection of the first radioactive fraction, adsorption on charcoal and subsequent elution with phenol-saturated water, paper chromatographic separation with butan-1-ol–acetic acid–water (50:12:25, by vol.) followed by paper electrophoresis in 0·1 M-sodium acetate–acetic acid buffer, pH 4·5.

The chromatographic mobilities of the final product in three different solvent systems were identical with those of an authentic sample of *p*-hydroxyphenylacetic acid *O*-sulphate. Electrophoretic mobilities of metabolite and authentic sample were also identical and both materials showed $\lambda_{max.}$ 265 mμ in presence of N-NaOH. After acid hydrolysis and recovery of the phenolic moiety via ether extraction, both materials gave products absorbing maximally at 295 mμ. Isotope dilution experiments in which the sulphated metabolite was recrystallized seven times in the presence of an authentic unlabelled sample of *p*-hydroxyphenylacetic acid *O*-sulphate yielded material of constant specific activity after two recrystallizations. The final recrystallized material moved as a single spot during paper electrophoresis and on paper chromatograms.

Intraperitoneal injection of synthetic *p*-hydroxyphenylacetic acid *O*-[^{35}S]sulphate was followed by rapid elimination of the compound in an unchanged form in the urine. This ester may therefore be presumed to represent the end-product of metabolism of TOS in the rat. The other main metabolite of TOS may be an intermediate on the metabolic pathway between TOS and *p*-hydroxyphenylacetic acid *O*-sulphate. The possibility that this second metabolite is *p*-hydroxyphenylpyruvic acid *O*-sulphate is now being investigated.

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Dodgson, K. S., Powell, G. M. & Tudball, N. (1960). *Biochem. biophys. Res. Commun.* **2**, 130.

(9) Changes in Two Microsomal Components accompanying Cell Differentiation.

By U. E. Loening. (*Department of Botany, University of Edinburgh, Edinburgh* 3)

The microsomes of growing root tips of pea and maize seedlings were investigated to study their role in cell differentiation (Bonner, 1959; Wright, 1960).

Root tips were cut into three serial segments, 1·6, 1·8 and 3·0 mm. long. The first includes the root cap and meristematic cells. The second and third show progressive stages of cell differentiation but no division. The segments were homogenized in 0·4 M-sucrose or in a medium at pH 6·9 containing 0·5 M-sucrose, 2·5 mM-2-amino-2-hydroxymethylpropane-1:3-diol, 4·0 mM-KH_2PO_4 and 10 μM-$MgSO_4$. After centrifuging at 30 000 *g*, the microsomes were sedimented at 140 000 *g*. The supernatants were removed, the tubes inverted into larger glass tubes, and centrifuged at up to 300 *g* for 3 min. This caused the pellets to divide: a clear, firm pellet

('heavy' microsomes) remained at the tip of each tube while a translucent yellowish pellet slid into the larger glass tube ('light' microsomes).

The pellets were analysed for RNA spectrophotometrically (Littlefield, Keller, Gross & Zamecnik, 1955) and for protein by a Kjeldahl micro-Conway method. The amount of 'heavy' microsomes, expressed as μg. of protein or RNA per cell, was highest in the meristematic segment and fell with age, while the amount of 'light' microsomes was close to zero in this region and increased with age. The ratio light microsomes:heavy microsomes in terms of protein content was 0·1–0·3:1 in the first segment, 1–3:1 in the second and 3–7:1 in the third. The ratio protein:RNA also varied with age, from 0·7:1 in the tip to 0·4:1 in the third segment in 'heavy' microsomes and 1·0:1 to 1·5:1 in 'light' microsomes.

The microsomes were examined by electrophoresis using the technique of Kohn (1958) and a citric acid–potassium phosphate buffer at pH 6·7. After ribonuclease treatment, the 'heavy' microsomes showed two nucleic acid components and the 'light' microsomes four or five. Much of the protein in the 'light' microsomes became insoluble but more components were obtained than from the 'heavy'.

The pellets are being examined by electron microscopy and with respect to the base components of the RNA (Heyes, 1960).

It is suggested that the changes observed are of significance in changes of protein synthesis accompanying differentiation.

Bonner, J. (1959). *Amer. J. Bot.* **46**, 58.
Heyes, J. K. (1960). *Proc. Roy. Soc.* B, **152**, 218.
Kohn, J. (1958). *Clin. chim. Acta*, **3**, 450.
Littlefield, J. W., Keller, E. B., Gross, J. & Zamecnik, P. C. (1955). *J. biol. Chem.* **217**, 111.
Wright, S. T. C. (1960). *Nature, Lond.*, **185**, 82.

(10) Inhibitor Studies on the Time Sequence of Nucleic Acid and Protein Synthesis in Mammalian Cells.

By J. Paul and A. Hagiwara. (*Department of Biochemistry, The University, Glasgow, W.* 2)

These investigations were undertaken to investigate the sequence of events in the synthesis of DNA, nuclear RNA, nuclear protein, cytoplasmic RNA and cytoplasmic protein. Continuous cultures of mouse subcutaneous fibroblasts, strain L, clone 929 (Sanford, Earle & Likely, 1948) were used. In one group of experiments DNA synthesis was inhibited by 0·1 μM-fluorodeoxyuridine and the synthesis of cell components was studied at intervals after its introduction. In another group of experiments cells were exposed to an inhibitory concentration of fluorodeoxyuridine for up to 48 hr. and the inhibition was then reversed by replacing the medium with one containing 0·1 mM-thymidine. Estimates of the rates of synthesis of the different components were obtained by (*a*) gross analysis of the cultures, and (*b*) measurement of the incorporation of ^{32}P or ^{14}C (as formate) for a standard time under standard conditions. Immediately after exposure of the cells to fluorodeoxyuridine the rate of synthesis of DNA and nuclear protein fell sharply. The synthesis of nuclear RNA continued for about 6 hr. and then declined. The synthesis of cytoplasmic RNA remained at a fairly high level for at least 12 hr. before declining and that of cytoplasmic protein began to fall later. By 36 hr., incorporation of tracer into all these compounds had fallen to a low level. When the inhibition was reversed by the addition of thymidine, synthesis of DNA recovered first, followed by nuclear RNA, nuclear protein, cytoplasmic RNA and cytoplasmic protein, in that order. In all cases an initial burst of activity was followed by a fall to a lower level. The highest rates of incorporation were reached in 2 hr. for DNA, $\frac{1}{2}$hr. for nuclear RNA, 8 hr. for nuclear protein, 12 hr. for cytoplasmic RNA and about 16 hr. for cytoplasmic protein. If the commencement of synthesis of these substances is dependent in any way on the prior synthesis of the others then the observations are compatible with current speculation about the transfer of information from DNA to cytoplasmic protein through nuclear RNA and cytoplasmic RNA.

Sanford, K. K., Earle, W. R. & Likely, G. D. (1948). *J. nat. Cancer Inst.* **9**, 229.

(11) 'Conjugate' Formation During the Metabolism of [4-^{14}C]progesterone by Female-Rat Liver *in vitro*.

By B. A. Cooke and W. Taylor. (*Department of Physiology, The Medical School, King's College, University of Durham, Newcastle upon Tyne*, 1)

In previous studies of the metabolism of progesterone *in vitro* the sensitivity of the analytical methods then available necessitated the use of tissue:steroid ratios of approximately 1000:1 (Taylor, 1954, 1955). The metabolism of [4-^{14}C]-progesterone of relatively high specific activity by female-rat-liver homogenate has now been investigated at tissue:steroid ratios of 56 000:1.

In the first series of experiments the steroid was dissolved in aqueous ethanol, the composition of the incubation medium being (values in brackets denote μmoles): progesterone (0·0143); ethanol (344); KH_2PO_4 buffer, pH 7·4 (120); KCl (150); $MgSO_4,7H_2O$ (15); nicotinamide (90); 250 mg. of

liver as homogenate; total vol. 3 ml. After 30 min. incubation in air at 37° reaction vessels were treated by the method of Taylor (1954). From 0–58% of initial radioactivity remained in the aqueous phase (pH 8·3) after hexane–chloroform extraction, indicating that 'conjugation' might have occurred. Therefore standard hydrolytic methods were applied to the aqueous solutions after the initial extraction. Incubation with limpet powder at pH 4·5, with neutralization before extraction, yielded more radioactivity; as much as 20% in experiments in which much 'conjugation' had occurred. Saccharate prevented the liberation of this radioactivity, suggesting that glucuronosides were formed during metabolism. After treatment of the enzyme-hydrolysed aqueous solutions with cold 2·2N-H_2SO_4 and then hot 1·1N-HCl followed by neutralization more radioactivity was extracted into hexane–chloroform.

Ethanol was found to inhibit 'conjugate' formation and in subsequent experiments was omitted from the incubation medium.

When DPN (0·74 μmole) and/or UDPglucose (0·32 μmole) were added to the incubation medium and vessels were gassed with O_2 'conjugation' was greatly increased. However radioactivity extractable after limpet-powder hydrolysis was only slightly increased, and the residual radioactivity in the aqueous solution could be recovered only after treatment with hot and cold acid. This suggests either that glucuronosides not hydrolysable by limpet powder were formed, or that addition of DPN and UDPglucose caused increased formation of 'conjugates' other than glucuronosides. Preliminary experiments indicate that more 'conjugation' occurs in O_2 than in air, irrespective of whether or not DPN or UDPglucose are added.

The financial support of the Medical Research Council is gratefully acknowledged.

Taylor, W. (1954). *Biochem. J.* **56**, 463.
Taylor, W. (1955). *Biochem. J.* **60**, 380.

(12) Progesterone Metabolism in the Cat.

By W. Taylor and T. Scratcherd. (*Department of Physiology, The Medical School, King's College, University of Durham, Newcastle upon Tyne* 2)

[4-^{14}C]Progesterone (0·83–1·39 mg.; $10·6 \times 10^6$ counts/min./mg.) has been administered intravenously over 10 sec. to three anaesthetized male cats, and bile and urine collected for 4–5 hr. by intubation of the gall and urinary bladders.

Excretion of radioactivity was greater in bile (30·5–67·1%) than in urine (0·5–0·7%) and was extremely rapid, 12·3–42·7% of the dose appearing in the bile within 2 hr. of injection. Bile samples collected over 1 or 2 hr. were analysed separately but, because of the low radioactivity content, the total urine sample from each animal was studied. Radioactivity extractable by ether after successive hydrolytic procedures was determined after washing extracts with 0·1N-NaOH and water until neutral and returning the washings to the original aqueous solutions before the next hydrolysis step. Results are expressed as percentage of counts in the sample analysed. The following definitions are used: 'free'—metabolites extractable before hydrolysis; 'glucuronosides'—substances extractable after incubation with limpet powder (Dodgson, Lewis & Spencer, 1953) [release of these metabolites was inhibited by saccharo-1→4-lactone (Levvy, Hay & Marsh, 1957)]; 'cold acid' metabolites—radioactivity released on standing at room temperature for 70 hr. after being made 2N in H_2SO_4 (Burstein & Lieberman, 1958); 'hot acid'—metabolites extracted after heating the acidified solutions in a boiling-water bath for 30 min.; 'aqueous residue'—radioactivity remaining water soluble after all hydrolytic procedures. Total recovery of radioactivity ranged from 80·9 to 97·6%.

In the bile only 0·2–0·6% 'free' radioactivity was present. The amount of 'glucuronoside' decreased with time after injection while 'cold acid' hydrolysed metabolites increased; e.g. in an experiment in which bile was collected in 4-hourly fractions 'glucuronosides' accounted for 40·1, 20·9, 13·5 and 10·8% of the radioactivity in successive samples, the corresponding values for 'cold acid' metabolites being 9·4, 13·2, 14·9 and 17·9%. 'Hot acid' hydrolysed metabolites showed no consistent change, but radioactivity in the 'aqueous residue' always increased with time after injection.

In the urine, excretion of radioactivity in the various fractions was fairly constant from animal to animal, approximate percentage values being: 'free', 1; 'glucuronosides', 5; 'cold acid', 6; 'hot acid', 9; 'aqueous residue', 70.

Financial support from the Medical Research Council is gratefully acknowledged.

Burstein, S. & Lieberman, S. (1958). *J. biol. Chem.* **233**, 331.
Dodgson, K. S., Lewis, H. I. M. & Spencer, B. (1953). *Biochem. J.* **55**, 253.
Levvy, G. A., Hay, A. J. & Marsh, C. A. (1957). *Biochem. J.* **65**, 203.

(13) Non-enzymic Transamination between Glycine and Glyoxylate.

By LAURA W. FLEMING and G. W. CROSBIE. (*Department of Biochemistry, The University, Glasgow, W. 2*)

Non-enzymic transamination reactions of the type

$$R'\cdot CH(NH_2)\cdot CO_2H + R\cdot CO\cdot CO_2H \rightleftharpoons R'\cdot CO\cdot CO_2H + R\cdot CH(NH_2)\cdot CO_2H$$

under physiological conditions have been described by Nakada & Weinhouse (1953) for limited cases where R = H. The equilibria of the reactions were completely in favour of glycine formation. No evidence has been obtained for non-enzymic transamination in the case where both R and R′ are alkyl derivatives.

In the course of a study of C_1-unit formation from glycine a facile non-enzymic transamination reaction between [^{14}C]glycine and glyoxylate has been observed. The reaction proceeds smoothly at pH 7 and 37°. The reaction is catalysed by hydroxyl ions and is inhibited at values of pH lower than 6. At pH 7 the reaction is catalysed by copper ions and inhibited by ethylenediaminetetra-acetic acid. The mechanism of the reaction at pH 7 is envisaged as involving a prototropic re-arrangement of the Schiff base glyoxylideneglycine induced by co-ordination complex formation. The significance of these results will be discussed in relation to the present concepts of the role of glyoxylate in the enzymic formation of formate from glycine.

Pyridoxal phosphate has been found to reverse the catalytic effect of copper ions on the non-enzymic transamination reaction between glycine and glyoxylate. This contrasts markedly with its pronounced catalytic influence on the alanine–pyruvate and glutamate–α-oxoglutarate systems. The mechanism of the involvement of pyridoxal phosphate in non-enzymic transamination reactions was discussed.

The glycine–glyoxylate non-enzymic transamination reaction proceeds with a slow disappearance of glycine and glyoxylate. Chromatographic evidence for the formation of β-hydroxyaspartic acid and pyruvate has been obtained. When the transamination reaction is carried out in the presence of pyridoxal phosphate, hydroxypyruvate is formed in addition to β-hydroxyaspartic acid. The mechanism of the formation of these artifacts will be discussed.

Nakada, H. I. & Weinhouse, S. (1953). *J. biol. Chem.* **204**, 831.

(14) Photofixation of Nitrogen and Photoproduction of Hydrogen by Thiosulphate During Bacterial Photosynthesis.

By D. I. ARNON, M. LOSADA, M. NOZAKI and K. TAGAWA. (*Laboratory of Cell Physiology, University of California, Berkeley, U.S.A.*)

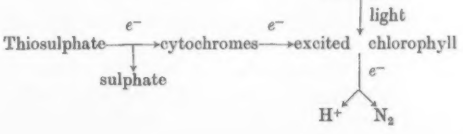
Photosynthetic bacteria fix N_2 or evolve H_2 in light (Kamen & Gest, 1949; Gest & Kamen, 1949). From the standpoint of the electron-flow theory of photosynthesis (Arnon, 1959, 1960), which excludes a photolysis of water, these phenomena could be viewed as resulting from a reduction of N_2 and H^+ respectively, by electrons donated by chlorophyll. The electrons come to chlorophyll via cytochromes from external electron donors and are raised to a reducing potential, at least equal to that of hydrogen gas, by photons absorbed during the primary photochemical act. This theory was tested by supplying thiosulphate as an electron donor to the photosynthetic sulphur bacterium, *Chromatium*.

Thiosulphate was found to reduce the cytochromes of *Chromatium*, that are oxidized by light. Photofixation of N_2 and photoproduction of H_2 were investigated in parallel experiments. Whole cells fixed N_2 with thiosulphate only in the light, and, most vigorously, when oxaloacetate was added as an amino group acceptor. N_2 fixation was inhibited by ammonia (cf. Kamen & Gest, 1949). In the absence of N_2, thiosulphate gave H_2 evolution in light. Gas evolution ceased when light was turned off and resumed when light was turned on again. No CO_2 source was supplied; KOH was present in the reaction vessels. Photoproduction of hydrogen was most vigorous at acid pH, was proportional to thiosulphate concentration (Losada, Nozaki & Arnon, 1960) and was inhibited by N_2 and ammonia.

Similar photofixation of N_2 and photoproduction of H_2 was obtained when thiosulphate was replaced by succinate. With succinate as electron donor, no additional amino group acceptor for photofixation of N_2 was necessary.

Cell-free *Chromatium* preparations fixed N_2 in the dark when supplied with H_2. These findings support the view that the role of light in photofixation of N_2 is to generate electrons with a reducing potential equal to H_2. N_2 fixation in whole cells and cell-free systems was confirmed with ^{15}N.

The following electron-flow mechanism for the photofixation of N_2 and photoproduction of H_2 is consistent with these findings:



We are indebted to Dr C. C. Delwiche for the determinations of ^{15}N.

Arnon, D. I. (1959). *Nature, Lond.*, **184**, 10.
Arnon, D. I. (1960). In *Light and Life*. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Gest, H. & Kamen, M. D. (1949). *Science*, **109**, 558.
Kamen, M. D. & Gest, H. (1949). *Science*, **109**, 560.
Losada, M., Nozaki, M. & Arnon, D. I. (1960). In *Light and Life*. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.

(15) The Effect of Protein Intake on the Ribonucleic Acid of Liver Cell Sap.

By ELIZABETH J. T. PROSSER, ANNETTE C. MALLINSON and H. N. MUNRO. (*Department of Biochemistry, The University, Glasgow, W.* 2)

The metabolism of RNA in the liver is sensitive to changes in the protein content of the diet. From isotopic and other data we have concluded that the rate of breakdown of a part of the liver RNA is regulated by the supply of amino acids from the diet (Clark, Naismith & Munro, 1957). There is evidence that this labile RNA is associated with endoplasmic reticulum and we have suggested that the protein content of the diet alters the relationship between reticulum-attached ribonucleoprotein and cell-sap RNA (Munro & Clark, 1960).

We have now examined the effect of dietary protein intake on the RNA of liver cell sap. This was prepared from rat livers homogenized in medium (Rendi & Campbell, 1959) and centrifuged at 105 000 *g* for 1 hr. The amino acid-activating enzymes and RNA were precipitated at pH 5·2. This fraction was then incubated with [DL-^{14}C]leucine and ATP (Rendi & Campbell, 1959), and the RNA examined for uptake of amino acid. Preparations from three groups of rats were studied (Clark *et al.* 1957). (*a*) Animals undergoing extensive loss of RNA from the cell, as the result of fasting overnight. (*b*) Animals from a similar group fed protein 1 hr. before killing; our previous evidence suggests a sudden cessation of breakdown under these circumstances. (*c*) Animals fed on a protein-deficient diet for several days before fasting overnight. This depletes the labile RNA of the liver cell and there is consequently no further loss of RNA on fasting. The amount of RNA in the cell sap was greatest in group (*a*), in which RNA breakdown was extensive, but it was significantly less active in accepting [^{14}C]leucine. This suggests that the cell-sap RNA was diluted by breakdown products from the reticulum.

The cell sap prepared from these three groups of animals was further fractionated by centrifuging at 105 000 *g* for 3 hr. more, resulting in a precipitate (post microsomal pellet) and a supernatant fluid. Diet no longer influenced the amount or amino acid uptake of the RNA of the supernatant fluid. The amount of RNA in the pellet varied considerably with diet, being greatest in the case of the rats of group (*a*), which were losing RNA from the cell, and least in the depleted animals of group (*c*). When pH 5 fraction was prepared from the pellet, the RNA showed some capacity to accept [^{14}C]leucine; the pellet RNA was most active when prepared from the protein-depleted animals. These findings suggest that the pellet RNA consists of two fractions, one of which varies in amount with diet but is unable to accept amino acids. Further evidence of differences in the heterogeneity of the pellet RNA on different diets was obtained by fractionation on 'Ecteola' columns (Goldthwaite, 1959).

We acknowledge gratefully a grant from the British Empire Cancer Campaign to carry out these experiments.

Clark, C. M., Naismith, D. J. & Munro, H. N. (1957). *Biochim. biophys. Acta*, **23**, 587.
Goldthwaite, D. A. (1959). *J. biol. Chem.* **234**, 3245.
Munro, H. N. & Clark, C. M. (1960). *Proc. Nutr. Soc.* **19**, 55.
Rendi, R. & Campbell, P. N. (1959). *Biochem. J.* **72**, 435.

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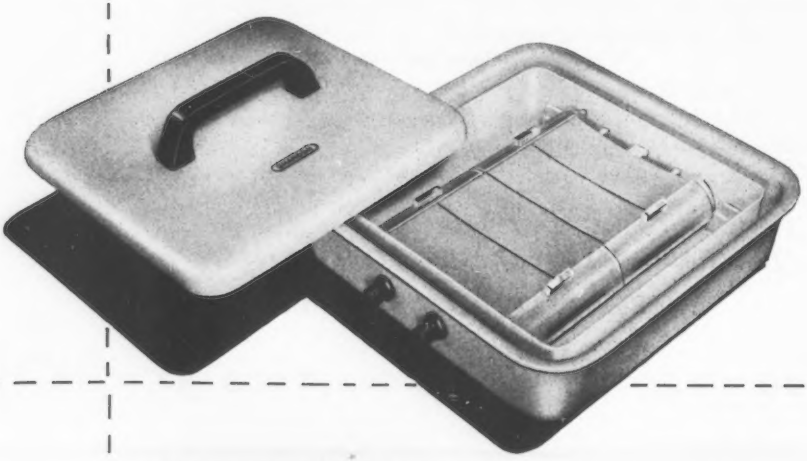

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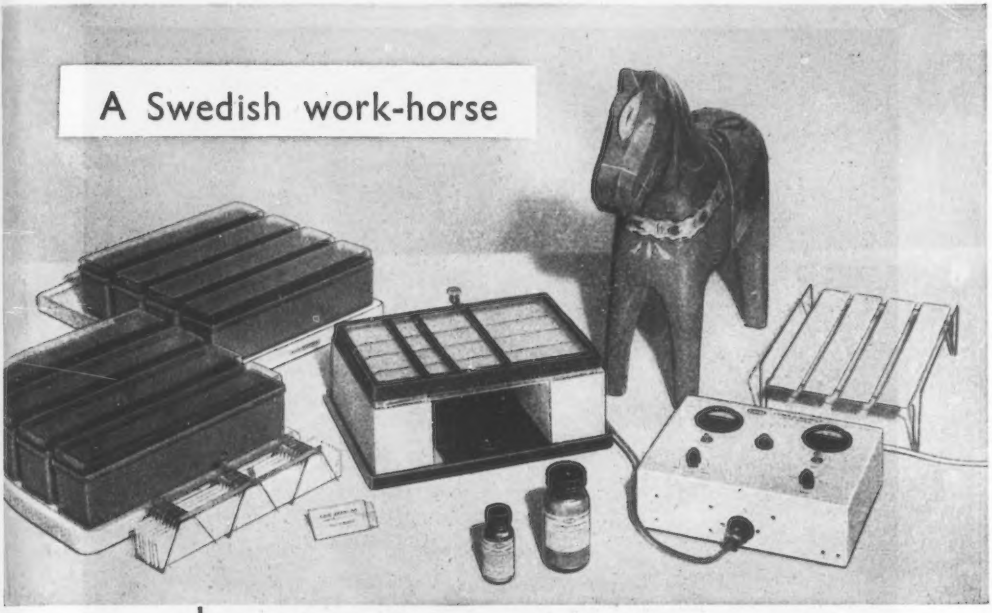

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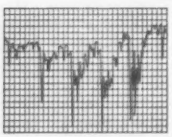

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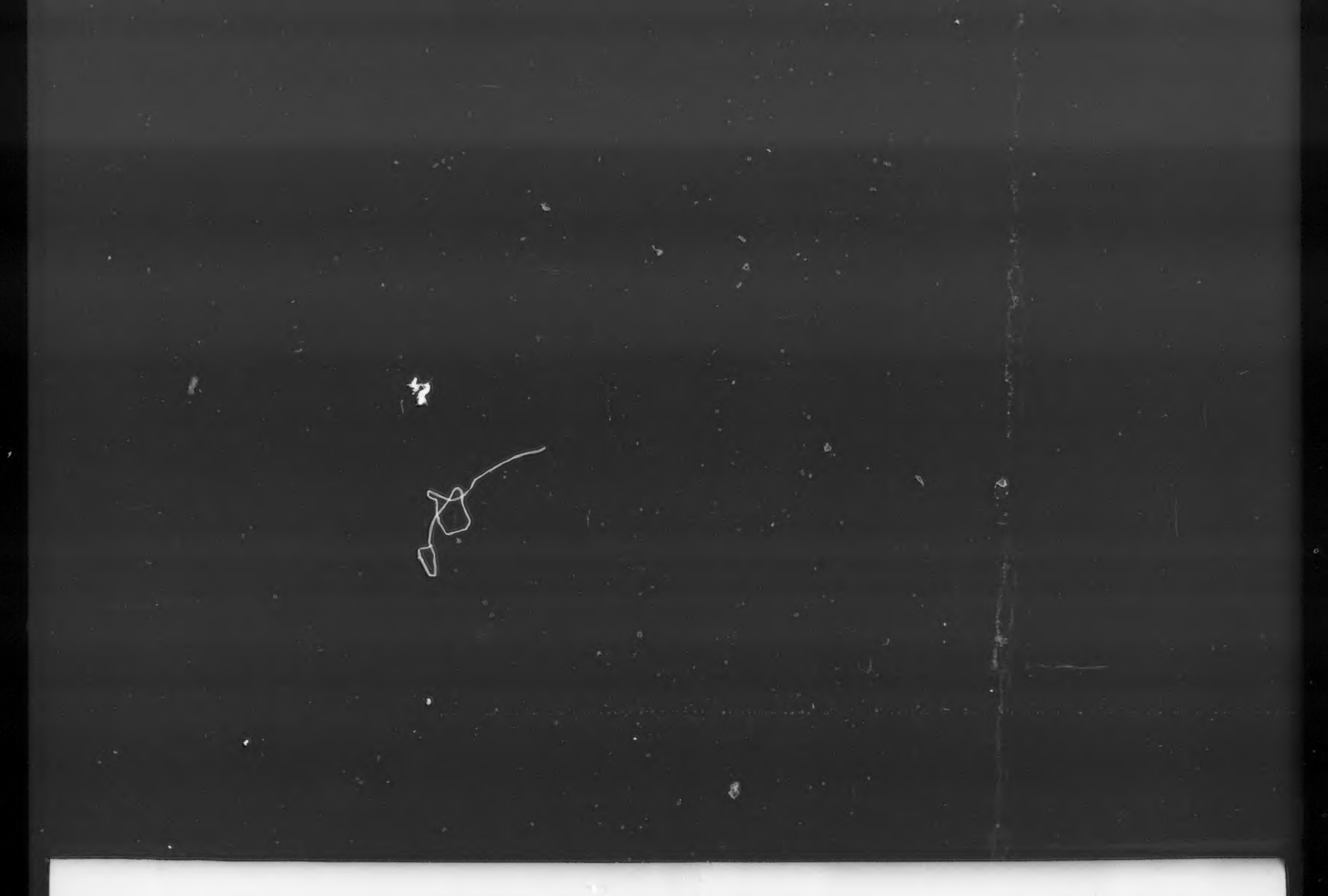

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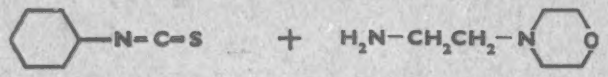

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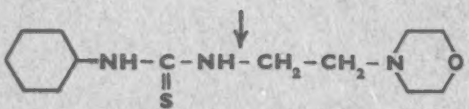

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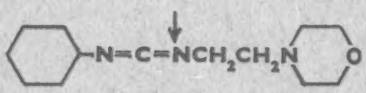

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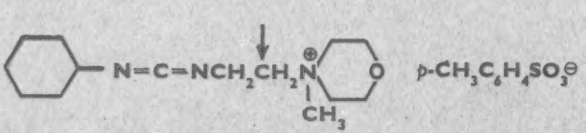

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